MECHANISMS OF GTP-DEPLETION INDUCED APOPTOSIS IN HIT-T15 INSULIN SECRETING CELLS

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A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

NATIONAL UNIVERSITY MEDICAL INSTITUTES

NATIONAL UNIVERSITY OF SINGAPORE

2003

Acknowledgments

My great appreciation goes to my supervisor, Dr. Li Guodong, for his invaluable supervision and encouragement throughout the course of this research project.

I thank all examiners (Dr. Marie-Veronique Clement at Dept of Biochemistry, NUS; Prof. Anjan Kowluru with Wayne State University, Detroit, MI, USA; and Prof. Noel G. Morgan at Peninsula Medical School, Plymouth, UK) for their critical reading of the thesis.

I am deeply indebted to my parents and my wife for their full-hearted support and understanding throughout my studies.

I am very grateful to all colleagues in my laboratory for their excellent technical assistance, encouragement and friendship.

I thank the National University of Singapore for the award of a research scholarship which gave me the opportunity to pursue my Ph. D studies.

Finally, I would like to say that my time as a post-graduate student at the NUS is a memorable and fulfilling stage in my life.

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Summary

Mycophenolic acid (MPA), selectively depletes intracellular guanine nucleotides by inhibition of IMP dehydrogenase, and induces apoptosis of islet β-cells. This study investigated the underlying mechanisms of this scenario.

As a calcium-dependent and GTP-modulated enzyme, tissue transglutaminase (tTG) may be involved in apoptosis by cross-linking intracellular proteins. Our results showed that MPA increased tTG activity (but not protein levels) in a dose- and time-dependent manner in close relationship with the induction of apoptosis. Co-exposure to either monodansylcadaverine or putrescine (tTG inhibitors) reduced MPA-enhanced tTG activity significantly, but failed to prevent the apoptosis. Similarly, lowering free Ca^{2+} concentrations by EGTA also did not improve cell viability, although most of the enhanced tTG activity was blocked. Importantly, a pan-caspase inhibitor, which entirely prevented apoptosis induced by MPA, did not block the enhancing effect of MPA on tTG activity, indicating that MPA may induce apoptosis and activate tTG independently. However, tTG inhibition was able to partially reverse the accompanied morphological changes. These findings suggest that tTG activation may be restricted to some terminal morphological changes, but may not play a critical role in the initiation of this kind of apoptosis.

This study also investigated the possible involvement of caspase(s) in GTP-depletion-induced apoptosis. MPA reduced progression of cell cycle from G1 phase into S and G2/M phases and induced apoptosis. The latter event was accompanied by a marked increase of caspase-2 activity and moderate activation of caspase-9 and -3. However, only caspase-2 activation preceded the appearance of apparent apoptosis. There was no change in activity of caspase-1, -4, -5, -6 and -8. Release of the mitochondrial

protein cytochrome *c* into cytosol was also observed at a late stage. Importantly, co-treatment of cells with a pan-caspase inhibitor blocked MPA-induced apoptosis in a dose-dependent manner. Moreover, a specific caspase-2 inhibitor, but not a caspase-3 inhibitor, was also capable of restoring cell viability. Interestingly, activation of caspase-2 appeared prior to caspase-3 activation. These results indicate that while activation of multiple caspases is involved in the execution of MPA-induced apoptosis of β-cells, caspase-2 plays the major role in the initiation of this kind of programmed cell death. These findings revealed a novel, caspase-2 mediated form of apoptosis that may be consequent to impaired mitogenesis.

Furthermore, the possible relationship between arrest of cell growth and activation of caspases during MPA-induced apoptosis of β-cells was examined, focusing on three important signalling molecules (p53, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$) which regulate cell cycle. $p21^{WAF1/CIP1}$ was significantly increased following MPA treatment. This phenomenon was closely correlated with the time-course of caspase activation under the same conditions. Interestingly, MPA-induced $p21^{WAF1/CIP1}$ was not mediated by p53, since p53 mass was gradually reduced during MPA treatment. In addition, the increment of $p21^{WAF1/CIP1}$ by MPA was further enhanced in the presence of a pan-caspase inhibitor, suggesting that an increase of $p21^{WAF1/CIP1}$ may occur prior to caspase activation. This notion of association of $p21^{WAF1/CIP1}$ accumulation with caspase activation and apoptosis was substantiated by using mimosine, a selective $p21^{WAF1/CIP1}$-inducer independent of p53. Mimosine, like MPA, also increased $p21^{WAF1/CIP1}$, promoted apoptosis and simultaneously increased activity of several caspases. In contrast to $p21^{WAF1/CIP1}$, a reduction in $p27^{KIP1}$ occurred in MPA-treated cells. These results indicate that $p21^{WAF1/CIP1}$ may act as an upstream signal by

blocking mitogenesis to activate caspases and in turn induce apoptosis during sustained GTP-depletion.

In conclusion, this work has demonstrated that GTP depletion-induced apoptosis of β-cells is mediated by activation of mainly caspase-2. In this process, $p21^{WAF1/CIP1}$ might be an important linking molecule between inhibition of mitogenesis and activation of caspases. However, tTG seems not to be a critical player in this scenario, though it may be involved in morphological alterations.

Abbreviations

All abbreviations are defined where they first appear in the text and some of the frequently used abbreviations are listed below.

AFC	7-amino-4-trifluoromethyl coumarin
BSA	bovine serum albumin
CARD	caspase recruitment domains
CDK	cyclin-dependent kinase
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid
CIP	Cdk inhibitory protein
CKI	cyclin-dependent kinase inhibitor
CPP32	cysteine protease p32
DED	death effector domains
DEVD-CHO	*N*-acetyl-Asp-Glu-Val-Asp-aldehyde
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol-bis [β-aminoethyl ether]-N, N, N'N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay

FACS	fluorescence-activated cell sorting
GN	guanine nucleotide
HRP	horseradish peroxidase
ICE	interleukin 1β-converting enzyme
ICH-1	ICE and CED-3 homolog 1
IFNγ	interferon γ
IL-1β	interleukin-1β
IMPDH	inosine-5'-monophosphate dehydrogenase
IP	immunoprecipitation
kDa	kilo-Dalton
Kip	kinase inhibitory protein
LDH	lactate dehydrogenase
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PI	propidium iodide
PMSF	phenylmethanesulfonyl fluoride
PVDF	polyvinylidene difluoride
RAIDD	receptor-interacting protein (RIP)-associated ICH-1/CED-3-homologous protein with a death domain

Rb	retinoblastoma protein
SDS	sodium dodecyl sulphate
TBS	Tris-buffered saline
TBS-T	TBS with 0.5% Tween-20
TEMED	N, N, N', N'-tetra methylthylene diamine
Tgase	transglutaminase
TNFα	tumor necrosis factor α
tTG	tissue transglutaminase
YVAD-CHO	*N*-acetyl-Tyr-Val-Ala-Asp-aldehyde
Z-VAD-FMK	benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

Publications

Journal Articles:

1. **JianXin Huo**, Rui-Hua Luo, Stewart A. Metz and GuoDong Li. (2002)**.** Activation of caspase-2 mediates the apoptosis induced by GTP-depletion in insulin-secreting (HIT-T15) cells. ***Endocrinology*** 143, 1695-1704

2. **Jianxin Huo**, Stewart A Metz and Guodong Li. (2003). Role of tissue transglutaminase in GTP depletion-induced apoptosis of insulin-secreting (HIT-T15) cells. ***Biochemical Pharmacology*** 66, 213-223

3. **Jianxin Huo**, Stewart A Metz and Guodong Li. (2003). p53-independent induction of $p21^{WAF1/CIP1}$ contributes to the activation of caspases in GTP-depletion induced apoptosis of insulin-secreting cells. ***Cell Death and Differentiation*** (2003 Sep 12 Epub ahead of print)

Conference Papers:

1. **Jianxin Huo**, Stewart A Metz and Guodong Li. (1999) Increment of tissue transglutaminase activity in apoptosis of insulin-secreting cells induced by sustained GTP depletion (at *the American Diabetes Association* 59^{th} *Scientific Sessions*, 22-26 June 1999, San Diego, CA, USA). The Abstract was published in ***Diabetes*** 48 (Suppl. 1): A248

2. **Jianxin Huo**, Stewart A Metz and Guodong Li. (2000) Induction of Caspase-2 Mediated Apoptosis of Insulin-secreting Cell due to GTP Depletion (at *the*

American Diabetes Association 60th Scientific Sessions, 9-13 June 2000, San Antonio, TX, USA). The Abstract was published in ***Diabetes*** 49 (Suppl. 1): A 259

3. **Jianxin Huo**, Stewart A Metz and Guodong Li. (2001) Activation of Caspases by Long-Term Treatment of High Fatty Acids in Insulin-Secreting β-Cells (at *the American Diabetes Association 61th Scientific Sessions,* 22-26 June 2001, Philadelphia, PA, USA). The Abstract was published in ***Diabetes*** 50 (Suppl. 2): A143

4. **Jianxin Huo**, Stewart A Metz and Guodong Li. (2001) A p53-independent $p21^{WAF1/CIP1}$ Pathway Probably Mediates Activation of Caspases in GTP-depletion Induced Apoptosis of Insulin-secreting Cells (at *the American Diabetes Association 61th Scientific Sessions*, 22-26 June 2001, Philadelphia, PA, USA). The Abstract was published in ***Diabetes*** 50 (Suppl. 2):A348

5. Jingsong Li, **Jianxin Huo**, Ruihua Luo, and Guodong Li. (2002). Role of the G-protein Rac1 in cell growth and insulin secretion in islet (INS-1) B-cells (at Research Symposium on Islet Biology, 25-28 October 2002, Falmouth, MA, USA)

Chapter 1

Introduction

1.1. Characteristics of apoptosis

1.1.1. Overview of apoptosis

Mechanisms that regulate cell death are essential for normal development and maintenance of homeostasis of the body. Individual cells may die either by accident or by design. Accidental cell death may be induced by lethal stimuli such as trauma or infection, leading to necrosis. Necrotic cells are characteristically swollen rather than shrunk. The loss of electrolytes and water balance following damage to the plasma membrane cause acidosis and osmotic shock, which distend cellular organelles such as mitochondria, endoplasmic reticulum (ER) and lysosomes. Eventually, inflammatory debris are released. Contrary to necrosis, death by design, which is termed **p**rogrammed **c**ell **d**eath (PCD), is a coordinated and regulated process (Lockshin and Williams, 1964; Lockshin, 1969, 1971). PCD includes apoptotic (Type 1) and non-apoptotic types; the latter can be further classed into vacuolar (Type 2), atrophic (Type 3), as well as differentiation to death (Fig. 1) (Bowen et al., 1998). Apoptotic cells are engulfed by neighboring cells before destruction and thus apoptosis will not induce inflammation.

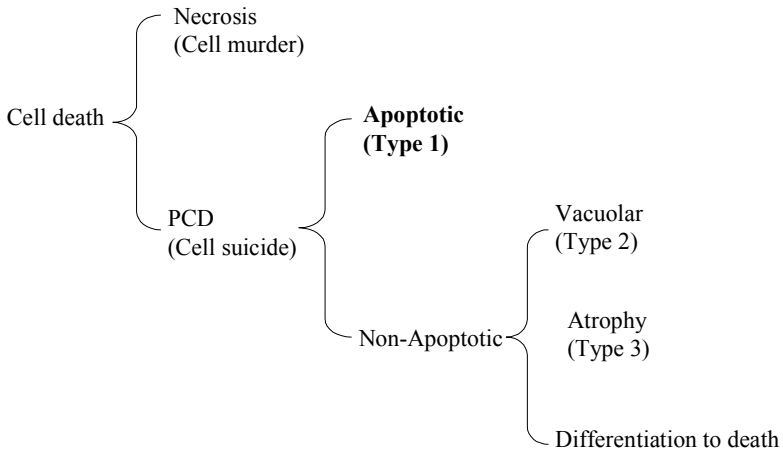

Fig. 1. Cell death classification

Apoptosis, a physiologically regulated destruction of supernumerary, misplaced or damaged cells with high specificity and efficiency, is critical for the normal development and function of multicellular organisms. Apoptosis is implemented through the activation of an evolutionarily conserved molecular programme intrinsic to all metazoan cells. Dysfunctions in the regulation or execution of apoptosis can contribute to a wide range of developmental abnormalities and diseases, including cancer (failure to undergo apoptosis), autoimmunity and degenerative disorders (excessive apoptosis) (Meier et al., 2000; Hengartner, 2000; Strasser et al., 2000).

1.1.2. Morphological and molecular changes of cellular organelles in apoptosis

As a physiological process for eliminating cells, apoptosis is distinct from necrosis which causes cell break-up and release of inflammatory debris. Apoptotic cells display some specific morphological changes, e.g. plasma membrane blebbing, nuclear condensation, and DNA fragmentation at nucleosomal intervals. These changes were first characterised by Kerr and colleagues in 1972 (Kerr et al., 1972). The more detailed features of apoptosis, including morphological changes and related molecular alterations, were defined in the following decades. Different subcellular compartments, such as cell membrane, cytoplasm, nucleus, mitochondria, ER, and Golgi apparatus, are implicated in apoptosis of diverse cells induced by a broad range of death stimuli.

1.1.2.1. Cytoplasm, nucleus and cell membrane

Apoptosis results in characteristic morphological features that are mostly evident in the nucleus, such as peripheral chromatin clumping, chromatin condensation, nuclear shrinkage, and the formation of apoptotic bodies. Cytoplasmic changes include cytoskeletal disruption, cytoplasmic shrinkage and condensation. These alterations are the results of biochemical and molecular changes, such as inter-nucleosomal DNA

cleavage (DNA ladder formation), RNA cleavage, post-translational modifications of nuclear proteins, and proteolysis of several polypeptides residing in the nucleus (Martelli et al., 2001). The changes in cell membrane during apoptosis include the externalization of phosphatidylserine (a phospholipid normally confined to the cytoplasmic face of the plasma membrane) to the outer membrane during apoptosis, and membrane blebbing which leads to the formation of cell membrane-bound vesicles or apoptotic bodies (Blankenberg et al., 2000).

1.1.2.2. Mitochondria

In contrast to nuclear shrinkage in apoptosis, 'swelling of mitochondria' may occur during the apoptotic processes. Enlargement of mitochondria induced by various apoptotic stimuli are classified into two categories: the swelling and the formation of megamitochondria (Matsuyama and Reed, 2000; Wakabayashi and Karbowski, 2001). Importantly, mitochondria play a critical role in the signaling of apoptosis in response to various stimuli. A series of events may occur, including: (1) changes in electron transport; (2) loss of mitochondrial transmembrane potential; (3) altered cellular oxidation-reduction; (4) participation of pro- and anti-apoptotic proteins of Bax/Bcl-2 family; and most importantly, (5) the release of caspase activators [such as cytochrome *c,* AIF (apoptosis-inducing factor) and SMAC (**s**econd **m**itochondria-derived **a**ctivator of **c**aspase)/Diablo (**d**irect **IA**P **b**inding protein with **lo**w pI)], and even caspases from the intermembrane space into the cytosol (Vander Heiden et al., 1997; Green and Reed, 1998; Shi, 2001). The exact mechanism by which these proteins release from mitochondria remains enigmatic. Eventually, the released cytochrome *c* and AIF will initiate a caspase-9 mediated apoptotic program (to be described in detail later) (Yang et al., 1997; Kluck et al., 1997; Susin et al., 1999b).

1.1.2.3. Endoplasmic Reticulum

ER is also a major place of integration of pro-apoptotic signaling or damage awareness. ER senses local stress through chaperones, Ca^{2+}-binding proteins and Ca^{2+} release channels, which might transmit ER's Ca^{2+} responses to mitochondria. ER also contains several Bcl-2-binding proteins, which may exert part of its cytoprotective effects within the ER (Ferri and Kroemer, 2001). Furthermore, ER is involved in activation of certain caspases. Nakagawa *et al* discovered that caspase-12 is localized in ER and activated by ER stress (such as disruption of ER calcium homeostasis or accumulation of excess proteins in ER), but not by membrane- or mitochondrial-targeted apoptotic signals (Nakagawa et al., 2000). Rao *et al* showed that ER stress also induces the expression of caspase-12, leads to the translocation of cytosolic caspase-7 to the ER surface, and mediates caspase-12 activation (Rao et al., 2001). Recently, Breckenridge *et al* reported an additional caspase-8 activation pathway in which procaspase-8L (the procaspase-8 isoform) is preferentially recruited by the complex of BAP31 (an integral protein of ER membrane and a substrate of caspase-8) at ER (Breckenridge et al., 2002).

1.1.2.4. Golgi apparatus

Fragmentation of Golgi apparatus was found as an apoptotic morphological change in a report by Dinsdale *et al* (Dinsdale et al., 1999). More interestingly, it was demonstrated that caspase-2 is localized in Golgi complex, where it cleaves golgin 160 which is a Golgi-localized macromolecule (Mancini et al., 2000). Recently, Lane *et al* found that GRASP65 (**G**olgi **r**eassembly **a**nd **s**tacking **p**rotein of 65 kDa), an important structural component required for maintenance of Golgi apparatus integrity, is cleaved specifically by caspase-3 (Lane et al., 2002). These results indicate that

Golgi complex may sense and integrate unique local changes and transduce pro-apoptotic signals through certain caspases (Mancini et al., 2000).

Taken all observations in this section together, we can envisage that apoptosis is a process which may not be restricted to a particular component of the cell. Various stimuli attack diverse cellular organelles and activate multiple apoptotic pathways which converge to a common machinery (e.g. caspase activation; being described later) of cell death.

1.2. Tissue transglutaminase and apoptosis

Some studies suggested that **t**issue **t**rans**g**lutaminase (tTG) might participate in the induction of apoptosis (Fesus and Thomazy, 1988; Fesus, 1998; Melino and Piacentini, 1998; Autuori et al., 1998). It has been reported that tTG selectively accumulates and its activity is markedly increased in cells undergoing apoptosis (Fesus and Thomazy, 1988; Fesus, 1998; Melino and Piacentini, 1998; Autuori et al., 1998). However, its exact role in apoptosis, albeit extensively studied in recent years (Fesus and Thomazy, 1988; Melino et al., 1997; Melino and Piacentini, 1998; Autuori et al., 1998), is still not clear.

1.2.1. Transglutaminase family

Transglutaminases (Tgases, EC 2.3.2.13) are a group of intracellular and extracellular thiol- and Ca^{2+}-dependent acyl transferases that catalyze the formation of an amide bond [irreversible ε- (γ-glutamyl) lysine or N, N-bis (γ-glutamyl) polyamine bonds] between γ-carboxamide groups of peptide-bound glutamine residues and the primary amino groups in various compounds, such as the ε-amino groups of lysine residues in certain proteins or the amino groups from polyamines (Fesus and Thomazy, 1988; Fesus, 1998; Melino and Piacentini, 1998; Folk et al., 1980; Folk, 1980; Fesus et al.,

1985; Piacentini et al., 1988). Generally, this kind of covalent isopeptide crosslink stabilizes tissues since it is stable and resistant to proteolysis, thereby increasing the resistance of tissue to chemical, enzymatic, and mechanical disruption (Greenberg et al., 1991).

At least seven different transglutaminases have been characterized and each of them has specific functions (Table 1) (Folk, 1980; Fesus and Thomazy, 1988; Aeschlimann and Paulsson, 1994; Aeschlimann et al., 1998).

Table 1. Transglutaminase family

Type	Abbreviation	M. W. (kDa)	Location	Function
Keratinocyte	Tgase 1, TG_K	92	Keratinocytes	Terminal differentiation events of keratinocytes (Kim et al., 1993; Kim et al., 1995)
Tissue	TGase 2, tTG, G_h, TG_C	80	Widely distributed in the cytosol of many cells and tissues	1. Signalling for GTP-binding protein coupled receptors (Nakaoka et al., 1994; Feng et al., 1996; Feng et al., 1999) 2. Multiple-function in post-translational modification of proteins (Piacentini et al., 2000) 3. Apoptosis (Melino and Piacentini, 1998; Autuori et al., 1998; Piacentini et al., 2000) 4. Differentiation (Hand et al., 1993)
Epidermal	Tgase 3, TG_E	77	Epidermis	Cross-link structural proteins forming the cornified cell envelope (Steinert and Marekov, 1995; Rice and Green, 1977)
Prostate	Tgase 4, TG_P	80	Anterior prostate gland of rodents	Semen coagulation (Aeschlimann and Paulsson, 1994; Lorand and Conrad, 1984)
Coagulation factor XIIIa		80	Blood plasma	Cross-link formation in fibrin (Lorand and Conrad, 1984; Aeschlimann and Paulsson, 1994)
Band 4.2 protein			Erythrocyte membrane	Structural component of the cytoskeleton (Cohen et al., 1993)
Novel	Tgase *X,* TG_x	81	Keratinocytes	Formation of the cornified envelope (Aeschlimann et al., 1998)

1.2.2. Tissue transglutaminase (tTG)

tTG (EC 2.3.2.13, or Tgase 2, TG_C) is a cytosolic protein with molecular weight of ~80 kDa (slightly different among species). Interestingly, tTG is a protein of dual function. On one hand, it possesses properties of a GTP-binding protein/GTPase (thus is also called G_h) which transduces the stimulation of certain receptors to phospholipase C_δ in some type of cells (Nakaoka et al., 1994; Feng et al., 1996; Im et al., 1997; Vezza et al., 1999). On the other hand, this protein has transamidating activity which causes cross-linking of specific substrates in cytosol and nucleus (Cooper et al., 2000). tTG is identified in pancreatic islet cells, endothelial and smooth muscle cells, heart muscle, medullary interstitial and mesangial cells of kidney (Fesus and Thomazy, 1988; Dvorcakova et al., 2002). It can also be induced under a variety of conditions in many other types of cells, suggesting its multiple functions (Piacentini et al., 2000).

Importantly, tTG activity is mainly regulated by intracellular levels of both Ca^{2+} and GTP; an increase of the former or a reduction of the latter stimulates its transglutaminase activity (Smethurst and Griffin, 1996; Melino and Piacentini, 1998; Zhang et al., 1998). Therefore, tTG activity may be suppressed in the presence of normal ambient GTP concentrations (mM range). Other factors such as redox potentials (glutathione disulfide and S-nitrosylation), polyamines and sphingosylphosphocholine may also modulate tTG activity (Melino and Piacentini, 1998).

The presence of transglutaminase activity in islets was firstly reported in 1983 (Gomis et al., 1983) and a number of studies attempted to understand its potential role in insulin secretion (Sener et al, 1984; Sener et al, 1985; Bungay et al, 1984; Gomis et al, 1986). It has been found that tTG may be involved in the membrane-mediated events

required for glucose-stimulated insulin release from beta-cells, since tTG inhibitors significantly inhibited glucose-stimulated insulin release from isolated rat islets (Bungay et al., 1986). Furthermore, a recent study reported that knockout of tTG in mice resulted in the loss of tTG and impaired glucose-stimulated insulin secretion leading to the development of type-2 diabetes (Bernassola et al., 2002). Therefore, tTG appears important for the maintainence of islet beta-cell function and insulin secretion (Dvorcakova et al., 2002).

1.2.3. Role of tTG in apoptosis

tTG is capable of cross-linking proteins and may prevent leakage of intracellular components, thereby reducing inflammation and autoimmunity. Some studies suggested that tTG might also participate in the induction of apoptosis (Fesus and Thomazy, 1988; Melino and Piacentini, 1998; Autuori et al., 1998; Fesus, 1998). Therefore, the role of tTG in the regulation of apoptosis is extensively studied in recent years.

Several *in vivo* and *in vitro* experimental systems have demonstrated a direct relationship between tTG (in both expression and activity) and apoptosis. It has been reported that tTG is selectively accumulated and its activity is markedly increased in cells undergoing apoptosis (Fesus and Thomazy, 1988; Melino and Piacentini, 1998; Autuori et al., 1998; Fesus, 1998; Uray et al., 2001). In addition, transcription of tTG mRNA is a consequence of apoptosis induction (Ou et al., 2000; Uray et al., 2001) and overexpression of tTG in various cell types enhances their susceptibility to apoptosis (Piredda et al., 1999; Piacentini et al., 2002). Interestingly, many of tTG-targeted proteins are also the substrates of caspases (Autuori et al., 1998). Moreover, an inhibition of tTG expression in human leukemic U937 cells undergoing apoptosis

induced by all-trans- retinoic acid (RA) prevents their death, although this kind of apoptosis is caspase-independent (Oliverio et al., 1999).

However, some recent studies indicated that tTG does not mediate apoptosis directly. In two reports (Nanda et al., 2001; De Laurenzi and Melino, 2001), the $tTG^{(-/-)}$ mice were viable and no major developmental abnormalities occurred. In addition, the induction of apoptosis in thymocytes and fibroblasts from these $tTG^{(-/-)}$ mice under a number of apoptotic conditions showed no significant difference from the wild-type cells. Furthermore, Boehm *et al* found that tTG possesses the ability of modifying **r**etino**b**lastoma (Rb) via transamidation, thereby providing protection against apoptotic insults (Boehm et al., 2002). These results indicated that tTG has very complex roles in apoptosis.

1.3. Caspases and apoptosis

In *Caenorhabditis elegans* (*C. elegans*), the ICE (**i**nterleukin 1β-**c**onverting **e**nzyme)/CED-3 (*C. elegans* **ce**ll **d**eath defective gene 3) encodes an aspartate-specific cysteine protease that is essential for apoptosis (Yuan et al., 1993; Xue et al., 1996). Subsequently, it was demonstrated that a large number of ICE/CED-3 related family of proteases also play key roles in mammalian apoptosis. For uniformity, these evolutionarily conserved cysteine proteases that cleave specific aspartic acid residues were termed caspases (Alnemri et al., 1996; Thornberry and Lazebnik, 1998). Caspases are not only responsible for the degradation of cellular substrates during the end stage of apoptosis, but also critical regulators of cell death initiation, playing critical roles in both the initiating and the executive phases of apoptosis. Different apoptotic signals converge on a common machinery of cell death by activating caspases (Strasser et al., 2000).

1.3.1. Caspase family

Caspases are localized in various subcellular compartments. At least 14 members (Table 2) have been identified. Based on the presence of a large prodomain at their N-terminal region and the substrate specificity (Strasser et al., 2000), caspases can be classed into two groups possessing different functions; large prodomain caspases (initiator) receiving apoptotic signals function as upstream signal transducers (e.g. caspase-1, -2, -8, -9), whereas short prodomain caspases (effector or executor) are activated by the former and operate as downstream amplifiers that cleave death substrates (e.g. caspase-3, -6, and -7) (Kumar, 1999; Earnshaw et al., 1999).

Table 2. Members of caspase family

Caspase type	Synonyms	M.W. (kDa)	Prodomain module	Activation adapter	Active site	Preferred peptide substrates	Prodomain/function
1	ICE	45	CARD-	CARDIAK (?)	QACRG	YEVD	Long Prodomain / Cytokine processors
2	Nedd1, ICH1	51	CARD	RAIDD/ CRADD	QACRG	VDVAD	Long Prodomain/ Apoptotic initiator
3	CPP32, YAMA, Apopain	32	-	-	QACRG	DEVD	Short Prodomain/ Apoptotic effector
4	TX, Ich2, ICErII	43	CARD-	-	QACRG	LEVD	Long Prodomain/ Cytokine processors
5	TY, ICErIII	48	-	-	QACRG	LEHD	Long Prodomain / Cytokine processors
6	MCH2	34	-	-	QACRG	VEID	Short Prodomain/ Apoptotic effector
7	MCH3, CMH, ICELAP3	35	-	-	QACRG	DEVD	Short Prodomain/ Apoptosis effector
8	FLICE, MACH	55	DED	FADD	QACQG	IETD	Long Prodomain/ Apoptotic initiator
9	MCH6, ICELAP6	45	CARD	APAF1	QACQG	LEHD	Long Prodomain/ Apoptotic initiator
10	FLICE2, MCH4	55	DED	FADD	QACQG	LEAD	Long Prodomain/ Apoptotic initiator
11		42	-	-		WEHD	Long Prodomain / Cytokine processors
12		50	-	-		WEHD	Long Prodomain / Cytokine processors
13	ERICE	43	-	-		WEHD	Long Prodomain / Cytokine processors
14	MICE	30	-	-		WEHD	Short Prodomain / Cytokine processors

1.3.2. Caspase activation

In general, initiator caspases act at the upstream of a proteolytic cascade in response to apoptotic stimulation, whereas downstream effector caspases at the are involved in the cleavage of a set of proteins, resulting in apoptotic death of the cell (Thornberry and Lazebnik, 1998).

All caspases are expressed as proenzymes (30 to 55 kDa) that contain three domains: an NH_2-terminal prodomain, a large subunit (~20 kDa), and a small subunit (~10 kDa). Each active form of caspases is derived from two pro-caspases, which produce a heterotetramer composed of the two large subunits and the two small subunits from proteolytic processing between these domains (Thornberry and Lazebnik, 1998). This kind of tetrameric enzyme contains two active sites at opposite ends of the molecule (cf. Fig. 2, p. 14). Interestingly, this activation process can be performed by caspases themselves. Studies have showed that caspase 1 through 10 all are capable of auto-processing (Earnshaw et al., 1999). The aggregation or oligomerisation of initiator caspase zymogens is sufficient to promote auto-processing/activation and, subsequently, trigger an avalanche of activation of other downstream effector caspases (Thornberry and Lazebnik, 1998).

1.3.3. Adaptor proteins for caspase activation

It is not entirely clear how the caspases with a large prodomain are activated, but their binding to adaptor molecules, which results in their oligomerisation and a series of autocatalysis and heteroproteolysis (Kumar, 1999; Earnshaw et al., 1999), is a critical step. Activation of initiator caspases by various apoptotic signals may be mediated by specific adaptors (Table 2 and Fig. 2). A good example is FADD/MORT1 (**F**as-**a**ssociating **d**eath **d**omain /**m**ediator **o**f **r**eceptor-induced **t**oxicity 1). It possesses a motif known as the **d**eath **e**ffector **d**omain (DED) which can interact with the DEDs in

the pro-domain of caspase-8. Subsequently, proteolytic processing by caspase-8 itself or other proteases releases active caspase-8 from the **d**eath-**i**nducing **s**ignaling **c**omplex (DISC) (Medema et al., 1997). The other end of FADD has an interaction motif called **d**eath **d**omain (DD) which binds to signalling molecules and receptors sending the death signal. Therefore, FADD acts as an adaptor by transducing the death signaling to caspase activation via its DD and DED. Another well described adaptor protein is RAIDD [receptor-interacting protein (**R**IP)-**a**ssociated **I**CH-1/CED-3-homologous protein with a **d**eath **d**omain]. RAIDD also has a DD (Duan and Dixit, 1997; Hofmann et al., 1997). Different to FADD, RAIDD does not possess a DED domain but a **ca**spase **r**ecruitment **d**omain (CARD) that binds to the CARD in the pro-domain of caspase-2 (Duan and Dixit, 1997; Thome et al., 1998; Kumar, 1999; Earnshaw et al., 1999). This process is similar to the binding of DEDs between FADD and caspase-8. Thus these adaptors, including Apaf-1 [**a**poptotic **p**rotease **a**ctivating **f**actor-1, binding to cytochrome *c*] for caspase-9 and CARDIAK [**CARD**-containing interleukin (IL)-1 beta converting enzyme (**I**CE) **a**ssociated **k**inase, interacting with the TNFR-associated factors] for caspase-1, possess both distinct domains responsible for their interactions with the upstream apoptotic molecules and with the prodomains of initiator caspases (Kumar, 1999; Earnshaw et al., 1999). Consequently, they may well be the targets regulated by other cellular signaling pathways.

1.3.4. Apoptosis signaling

Three apoptosis signalling pathways are summarized in Fig. 2. Two major caspase-mediated apoptosis pathways have been well established (Kumar, 1999; Earnshaw et al., 1999). One involves the membrane death receptors [such as Fas antigen (CD95 or APO-1) and **t**umor-**n**ecrosis-**f**actor **r**eceptor (TNFR)] which operate apoptotic death with caspase-8 acting as the upstream initiator in the cascade of caspase activation

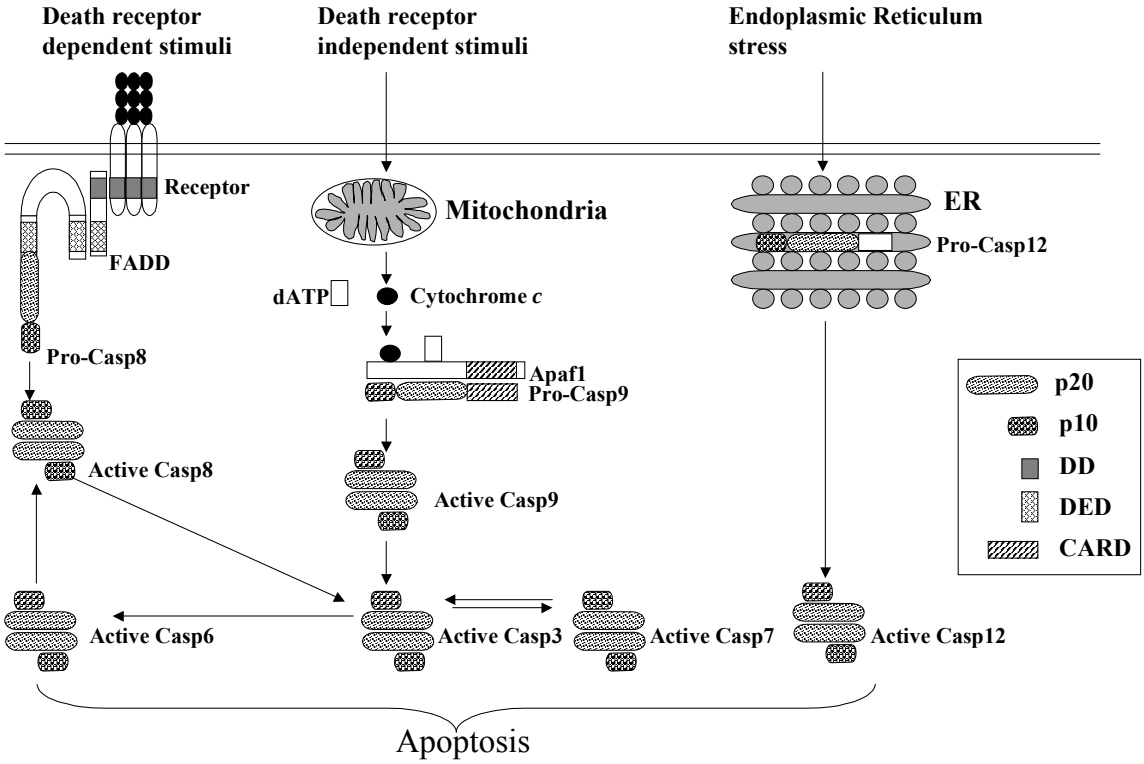

Fig. 2. Apoptosis signalling pathways

(Kumar, 1999; Earnshaw et al., 1999). Another cascade (initiated by caspase-9) of caspase activation is (independent of death receptor) involved in the apoptosis due to mitochondria damage (releasing cytochrome *c*) such as that caused by radiation and toxic drugs (Li et al., 1997b; Green and Reed, 1998; Ito et al., 1999). More recently it has been reported that caspase-12 mediates the apoptosis induced by ER stress by agents that release calcium from intracellular stores (Nakagawa et al., 2000), while caspase-11 is initially activated in the inflammatory LPS-induced cell death (Wang et al., 1998).

1.3.5. Caspase substrates

Many cellular substrates that undergo cleavage by caspases during apoptosis have been reviewed by Earnshaw *et al* (Earnshaw et al., 1999), including protein kinases, nuclear proteins, cytoskeletal proteins, proteins involved in signal transduction, cell

cycle regulation and DNA metabolism/repair, as well as caspases themselves. The plethora of caspase substrates indicates that caspases play an executive role in the destruction of the cell.

However, growing evidence has showed that non-caspases, such as cathepsins, calpains, granzymes and the proteasome complex, may also mediate and promote cell death (Johnson, 2000).

1.3.6. Caspase inhibitors

The activity of caspases is regulated by their adaptors and other molecules such as the Bcl-2 family and FLIP (**FLI**CE-inhibitory **p**rotein). In addition, some inhibitors directly interact with the proteases. For instance, viruses carry cell death inhibitors by binding to the activated caspases to block the response of host cells. The viral and cellular caspase inhibitors include **c**ytokine **r**esponse **m**odifier ***A*** (CrmA), **p**rotease **i**nhibitor 9 (PI-9, also known as granzyme B inhibitor), p35, and **i**nhibitor of **a**poptosis **p**roteins (IAPs) (reviewed by Ekert et al.). Importantly, many synthetic caspase inhibitors have been developed based on the substrate cleavage sites of the caspases for pharmacological and experimental use (Ekert et al., 1999).

1.4. Cell cycle and apoptosis

1.4.1. Cell cycle regulation: co-ordination of cyclins, CDKs and CKIs

Cell cycle is co-ordinated by three families of molecules: cyclins, **c**yclin **d**ependent **k**inases (CDKs), and **c**yclin dependent **k**inase **i**nhibitors (CKIs) (Fig. 3) (Roberts et al., 1994). CDKs, the central players which control the initiation, progression and completion of the cell cycle, are a group of protein kinases which must assemble into a holoenzyme with a cyclin subunit to become catalytically active. The function of the cyclin-CDK complex is regulated by phosphorylation of CDKs at different positions

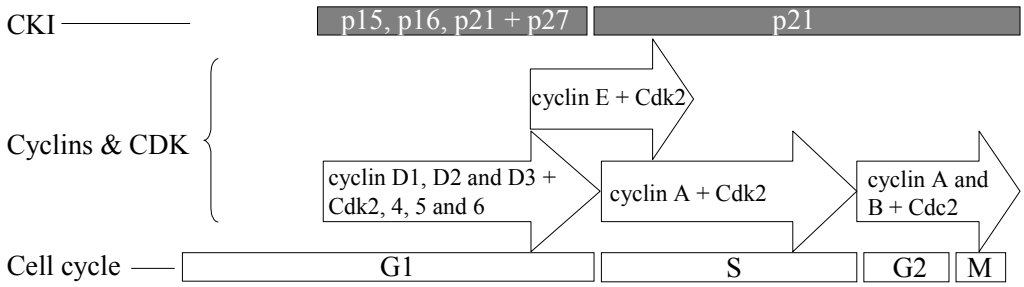

Fig. 3. Cell cycle regulators

(activation) and by proteolysis of cyclins (inactivation) (Roberts et al., 1994). Active CDKs phosphorylate retinoplastoma protein (Rb) and histones, relieving cell cycle from blockade and promoting cell cycle progression. In mammalian cells, CDK-2, -3, -4 and –6 all function in G1 and /or S phase while the cdc2 (CDK1) appears to be restricted to mitosis (Sherr, 1993). The expression and role of each cyclin are also distinct in different phases of cell cycle. Cyclin B is critical for mitosis (Takizawa and Morgan, 2000), whereas cyclin A is essential during S phase and cyclin D and E are necessary in regulating the progression through G1 (Roberts et al., 1994; Takizawa and Morgan, 2000). During cell cycle, cells express D-type cyclins (D1-D3) in mid G1, cyclin E in late G1, cyclin A in S phase, and B-type cyclins in late G2 and mitosis. To antagonize the action of CDK-cyclin complexes in cell cycle, mammalian cells express two families of CKIs to bind and inhibit cyclin-CDK complexes: the p16 family (INK4) and the $p21^{WAF1/CIP1}$ family (Roberts et al., 1994; Sherr and Roberts, 1999). The former family, including $p15^{INK4b}$ (Hannon and Beach, 1994), $p16^{INK4a}$ (Serrano et al., 1993), $p18^{INK4c}$(Guan et al., 1994; Hirai et al., 1995) and $p19^{INK4d}$(Hirai et al., 1995; Guan et al., 1996), specifically inhibits cyclin-D-CDK complexes, whereas the latter family (including $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$) is capable of inactivating all known cyclin-CDK complexes. Expression of these two families of CKIs is induced by inhibitory regulators of cell cycle and occurs during normal state of growth arrest (Sherr and Roberts, 1999).

1.4.2. Cell cycle and apoptosis

Apoptosis may occur at any stages of the cell cycle. Apoptosis is regarded as a process intimately linked to the cell cycle, since it is most frequently found in proliferating tissues and artificial manipulation of the cell cycle can either prevent or potentiate apoptosis (Meikrantz and Schlegel, 1995). Therefore, the transition from G1 to S phase is regarded as a crucial point for deciding the cell growth and apoptosis, because either normal division or mitotic catastrophe will be carried out depending on DNA synthesis in this state (Meikrantz and Schlegel, 1995). Particularly, cell progression from late G1 into S phase is regulated by p53 and by activation of CDKs in mammalian cells (El Deiry et al., 1993; Roberts et al., 1994; Meikrantz and Schlegel, 1995).

The growth suppressor p53 is a tightly regulated transcription factor that can induce either cell cycle arrest or apoptosis dictated by its expression levels in response to various stresses (Chen et al., 1996; Benchimol, 2001). In addition, p53 is particularly important for protecting cells when DNA is damaged by radiation, chemicals or viral infection (Cox and Lane, 1995). In this context, p53 is involved in the control of cell cycle in G1 and G2 phases (O'Connor, 1997; Mercer, 1998; Bunz et al., 1998; Taylor and Stark, 2001) by activating different target genes, such as $p21^{WAF1/CIP1}$ (El Deiry et al., 1993), GADD45 (GADD, **g**rowth **a**rrest and **D**NA **d**amage-inducible) (Kastan et al., 1992) and 14-3-3σ (Taylor and Stark, 2001; Benchimol, 2001).

1.4.3. $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and cell cycle

$p21^{WAF1/CIP1}$ is a general inhibitor of cyclin-CDK complexes (Xiong et al., 1993). This protein is also given other names: WAF1 (**w**ild-type p53-**a**ctivated **f**ragment 1) (El Deiry et al., 1993), CIP1 (**C**dk-**i**nteracting **p**rotein 1) (Flores-Rozas et al., 1994; Harper et al., 1995), SDI1 (**s**enescent cell-**d**erived **i**nhibitor 1) (Noda et al., 1994) or

MDA-6 (**m**elanoma **d**ifferentiation-**a**ssociated protein). It has been reported that $p21^{WAF1/CIP1}$ can act as either a downstream target/effector of p53 or independent of p53 (El Deiry et al., 1994; Alpan and Pardee, 1996) to coordinate the cellular responses to inhibitory growth signals (El Deiry et al., 1993; Chiarugi et al., 1994; Gartel et al., 1996; Boulaire et al., 2000; Benchimol, 2001). An increase of this CKI molecule induces cell cycle arrest at G1 and blocks entry into S phase by inactivating CDKs or/and by inhibiting the activity of **p**roliferating **c**ell **n**uclear **a**ntigen (PCNA).

$p27^{KIP1}$ is another CKI and shares homology at the N-terminal CDK-inhibitory domain with $p21^{WAF1/CIP1}$ (Polyak et al., 1994; Toyoshima and Hunter, 1994). It interacts with the cyclin/CDK complexes and suppresses the kinase activity of cyclin A-CDK2, cyclin D-CDK4, and cyclin E-CDK2 (Toyoshima and Hunter, 1994; Polyak et al., 1994) by preventing their activation and inhibiting previously activated complexes (Toyoshima and Hunter, 1994; Polyak et al., 1994). $p27^{KIP1}$ plays a role in the regulation of the progression through G1 and obstructs G1/S transition (Koff and Polyak, 1995; Zetterberg et al., 1995; Alessandrini et al., 1997; Moller, 2000). Different to $p21^{WAF1/CIP1}$, induction of $p27^{KIP1}$ expression is independent of p53 (An et al., 1998; Sowa et al., 1999; Zi and Agarwal, 1999; Patel et al., 2000; Pellizzaro et al., 2001).

1.5. GTP depletion and apoptosis

1.5.1. GTP biosynthesis and IMP dehydrogenase (IMPDH)

Normal intracellular levels of GTP (in the mM range) and other **g**uanine **n**ucleotides (GNs) modulate many biochemical reactions and their concentrations must be maintained at a critical level for normal cell signalling, metabolism, proliferation, differentiation, and other functions (Pall, 1985). GTP is necessary for the activation of

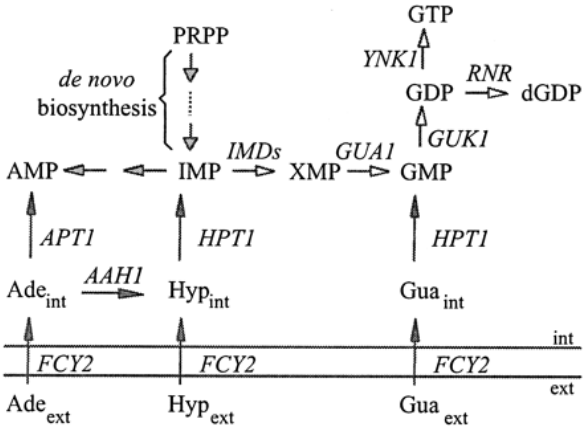

Fig. 3. Schematic representation of purine nucleotides *de novo* and salvage pathways. *Solid lines* represent the plasma membrane. The following abbreviations are used: *Ade*, adenine; *ext*, extracellular medium; *Gua,* guanine; *Hyp,* hypoxanthine; *int,* intracellular compartment; *PRPP*, 5-phosphoribosyl-1-pyrophosphate. Gene names are *italicized* and encode the following enzymatic activities: *AAH1*, adenine deaminase; *APT1*, adenine phosphoribosyltransferase; *FCY2*, purine cytosine permease; *GUA1*, guanosine-5'-monophosphate synthetase; *GUK1*, guanosine-5'-monophosphate kinase; *HPT1*, hypoxanthine-guanine phosphoribosyltransferase; *IMDs*, inosine-5'-monophosphate dehydrogenases; *RNR*, ribonucleotide reductase; *YNK1*, nucleoside-5'-diphosphate kinase. (This figure is adapted from the paper of Mafalda Escobar-Henriques and Bertrand Daignan-Fornier. *J. Biol. Chem. (2001), Vol. 276, Issue 2, 1523-1530)*

various GTP-binding proteins (G-proteins), for macromolecular synthesis (such as DNA, RNA and proteins), and is involved in signal transduction and exocytotic secretion (such as insulin release) (Jayaram et al., 1999; Metz and Kowluru, 1999; Metz et al., 2001). Moreover, GTP may be required for some other functions, such as ATP synthesis, calcium mobilization, microtubule assembly, vesicle traffic and protein kinase regulation (Metz et al., 1992).

Inosine 5'-monophosphate dehydrogenase (IMPDH, EC 1.1.1.205) is a rate-limiting enzyme in the de novo synthesis of GTP and other GNs (Metz and Kowluru, 1999; Allison and Eugui, 2000; Metz et al., 2001) (Fig 4). IMPDH catalyzes the NAD-dependent conversion of **i**nosine 5'-**m**ono**p**hosphate (IMP) to **x**anthine 5'-**m**ono**p**hosphate (XMP), which is further converted to **g**uanosine **m**ono**p**hosphate (GMP) by GMP synthase and, subsequently, to **g**uanosine **di**(**p**)hosphate (GDP) by GUK1, and finally to GTP by YNK1 (Metz and Kowluru, 1999; Jayaram et al., 1999; Allison and Eugui, 2000; Escobar-Henriques and Daignan-Fornier, 2001). The only alternative pathway for GTP biosynthesis is through the salvage of guanine to GMP by **h**ypoxanthine-guanine **p**hospho**r**ibosyl**t**ransferase (HPRT). The relative contributions

of the *de novo* and salvage pathways to GTP biosynthesis in different tissues and cell types have not been definitively determined. However, both the pathophysiology of inherited disorders of enzymes and the therapeutic effects of selected pharmacological inhibitors of these enzymes are well investigated to date (Gu et al., 2002).

There are two IMPDH isoenzymes, termed type I and type II. They are 84% identical at the amino acid level and possess indistinguishable catalytic activity. However, the regulation of expression of the two IMPDH genes differs dramatically. The increased IMPDH activity observed in replicating or neoplastic cells is largely due to increased expression of the type II IMPDH mRNA, whereas expression of the type I gene is relatively unaffected by cell proliferation or transformation (Nagai et al., 1991; Nagai, 2002). Type I IMPDH is constitutively expressed, whereas type II is upregulated in proliferating cells (due to higher rate of GTP production required for DNA synthesis), and highly down-regulated in terminally differentiated cells (due to relatively lower GTP synthesis for "housekeeping" and RNA synthesis) (Nagai et al., 1991; Carr et al., 1993; Natsumeda and Carr, 1993; Yalowitz and Jayaram, 2000). IMPDH gene expression is regulated inversely by a posttranscriptional nuclear event in response to fluctuations in the intracellular level of GNs (Azim et al., 1996).

Interestingly, IMPDH may be a rate-determining factor for p53-dependent cell growth regulation (Liu et al., 1998a). Sherley *et al* have demonstrated that p53 not only down-regulates IMPDH expression (Sherley et al., 1995; Liu et al., 1998a), but also selectively reduces IMPDH enzymatic activity (Sherley, 1991; Stadler et al., 1994). Recently, Kelly et al showed that supplement of guanosine reverses p53 increment in apoptosis induced by ischemic injury in tubular cells, whereas a specific inhibitor of p53 (Pifithrin-alpha) mimics the effects of guanosine to prevent apoptosis and protect renal function. (Kelly et al., 2003).

1.5.2. IMPDH inhibitors

Several structurally distinct IMPDH inhibitors have been identified. Some belong to the family of nucleoside compounds (including tiazofurin, selenazofurin, and benzamide riboside) which are anabolized to IMP analogues are able to inhibit IMPDH. Mizoribine (MZ) is also a competitive inhibitor of IMPDH. Contrary to MZ, **m**yco**p**henolic **a**cid (MPA) is a non-nucleoside IMPDH inhibitor showing uncompetitive inhibition with a Ki of 156 nM versus NAD (Carr et al., 1993; Halloran 1996, Yalowitz and Jayaram, 2000).

Since IMPDH has higher activity in proliferating than in quiescent cells, specific inhibitors of IMPDH have been widely used to treat cancers and psoriasis and more recently to prevent transplantation rejection (Jackson RC, 1975; Natsumeda et al., 1988; Luchetti et al., 1999). Many inhibitors of IMPDH are used clinically as antiviral (e.g. ribavirin), antitumor (e.g. tiazofurin, selenazofurin, and benzamide riboside), and immunosuppressive (e.g. MPA) drugs (Yalowitz and Jayaram, 2000).

These specific inhibitors interfere with cell proliferation and differentiation (Grusch et al., 1999; Jayaram et al., 1999). GTP depletion (down to μM ranges) by inhibition of IMPDH alone is capable of blocking RNA-primed DNA synthesis in the presence of all other rNTPs and dNTPs, including dGTP (Cohen et al., 1981). Generally speaking, growth inhibition by GTP depletion is a conserved pathway from Bacillus to humans (Yalowitz and Jayaram, 2000). In addition, GTP depletion may induce apoptosis as demonstrated in several types of cells (Catapano et al., 1995; Li et al., 1998; Metz and Kowluru, 1999; Hauser et al., 1999; Jayaram et al., 1999; Cohn et al., 1999; Yalowitz and Jayaram, 2000). Thus GTP depletion is a potent signal for arrest of cell proliferation and induction of apoptosis.

1.5.3. Mycophenolic acid (MPA)

Gosio firstly isolated MPA from a *Penicillium* culture in 1896. Alsberg and Black confirmed this work and gave the substance its current name in 1913. Raistrick *et al* illustrated the MPA structure in 1952 (Fig 5). MPA was found to have limited antibacterial and antifungal activity by some researchers including Abraham (in 1945), Florey, Gilliver and Jennings (in 1946). Importantly, Carter found that MPA has a potent antimitotic effect in mammalian cells and demonstrated that it is an effective antitumor agent (Williams et al., 1968; Carter et al., 1969). Recently, MPA is also widely used as a selective potent immunosuppressant to control cellular and antibody-mediated rejection in host tissues (Lanford et al., 2001). This is because antigen-activated B and T cells are highly dependent on purine *de novo* synthesis, whereas most other cells are capable of utilizing a salvage pathway.

MPA is a specific IMPDH inhibitor (Allison and Eugui, 2000). MPA depletes cellular GNs, leading to partial reduction in RNA synthesis and drastic inhibition of DNA synthesis; these inhibitory effects can be entirely reversed by provision of guanine or guanosine (but not by hypoxanthine, xanthine, adenine or adenosine) in a non-competitive manner (Franklin and Cook, 1969; Lowe et al., 1977; Nguyen and Sadee, 1986; Hauser et al., 1999; Digits and Hedstrom, 2000). Depletion of GNs by MPA

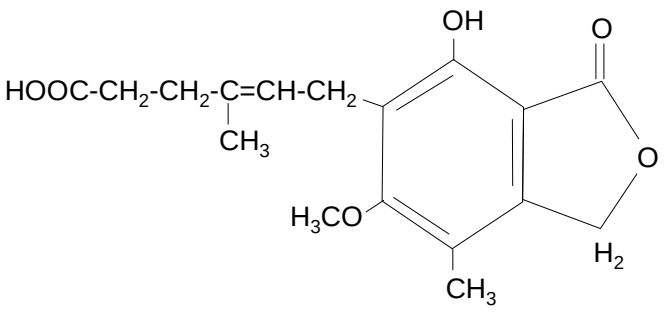

Fig 5. **Structure of mycophenolic acid**

may result in growth arrest and induction of apoptosis (Franklin and Cook, 1969; Metz and Kowluru, 1999; Yalowitz and Jayaram, 2000; Allison and Eugui, 2000; Allison, 2000).

Type II IMPDH is more sensitive to inhibition by MPA than the type I isoform (Luchetti et al., 1999). Of existing IMPDH inhibitors, MPA is the only one that has been shown to have preferable inhibition to the type II isoform of IMPDH (Yalowitz and Jayaram, 2000), with a 4.8- fold lower Ki than the type I (Allison and Eugui, 2000).

1.5.4. IMPDH in β-cells

Metz *et al* found that IMPDH expression is modulated by GN levels in insulin secreting cells (Metz et al., 2001). IMPDH is involved in both secretory and proliferation processes in β-cells (Li et al., 1998; Metz and Kowluru, 1999; Metz et al., 2001). It has been demonstrated that short-term depletion of GTP by using IMPDH inhibitors altered membrane ion fluxes and inhibited insulin secretion in islet β-cells (Metz et al., 1992; Meredith et al., 1997; Detimary et al., 1997; Komatsu et al., 1998; Metz and Kowluru, 1999; Li et al., 2000). The latter effect was probably due in part to the interference with the activation of phospholipase C and certain G-proteins (Kowluru et al., 1996). On the other hand, long-term inhibition of IMPDH arrested β-cell growth and triggered apoptosis (see below). Thus sufficient IMPDH activity (and thereby, availability of GTP) is important for the maintenance of insulin secretion and replication capability in β cells.

1.5.5. MPA and apoptosis of insulin secreting cells

A balance between β-cell proliferation and programmed cell death is important for the maintenance of β-cell mass necessary for secreting insulin to meet the body's requirement. There is evidence for a role of IMPDH in maintaining this balance. For

instance, interleukin-1β caused a reduction of GNs (Meredith et al., 1996) and apoptosis (Iwahashi et al., 1996) in islet β-cells.

Induction of apoptosis of β-cells (HIT-T15 cells, INS-1 cells and intact adult rat islets) by prolonged depletion of cellular GTP by two structurally-distinct IMPDH inhibitors (MPA and mizoribine) was first reported by Li *et al* (Li et al., 1998). HIT-T15 is a β-cell line derived from Syrian hamster islets transformed by SV40, whereas INS-1 cells were derived from radiation-induced rat β-cell tumors (Asfari et al, 1992). Noticeably, HIT-T15 cells are more sensitive to the inhibitory effects of MPA on GN synthesis than INS-1 cells or islets cells, due to a weak salvage pathway in the former cells (Meredith et al., 1997).

In the study by Li et al, sustained depletion of GTP caused inhibition of mitogenesis, decrement of cell number, DNA, protein content, and decline of metabolic viability in β-cells. These cells revealed typical apoptotic characters, such as apoptotic bodies, marginated chromatin, cytosolic vacuolization, loss of microvilli, and DNA fragmentation as assessed morphologically by electron microscopic observations (Fig. 6) and biochemically by DNA fragmentation (laddering). In addition, this kind of cell death was almost totally prevented by supplementation with guanosine, but not with adenosine, indicating that this is a specific effect of GTP depletion. However, the apoptosis induced by GTP depletion is not mediated by dGTP reduction because both low (30 μM) and high (500 μM) concentrations of exogenous deoxyguanosine, which could restore the dGTP content of cells even at 25 μM, failed to prevent the effect of MPA on cell death. How GN-depletion inhibits mitogenesis and induces apoptosis was unclear, although it might be due to the combined effects of inhibition of primer RNA biosynthesis and the interference with the function of some small G-proteins.

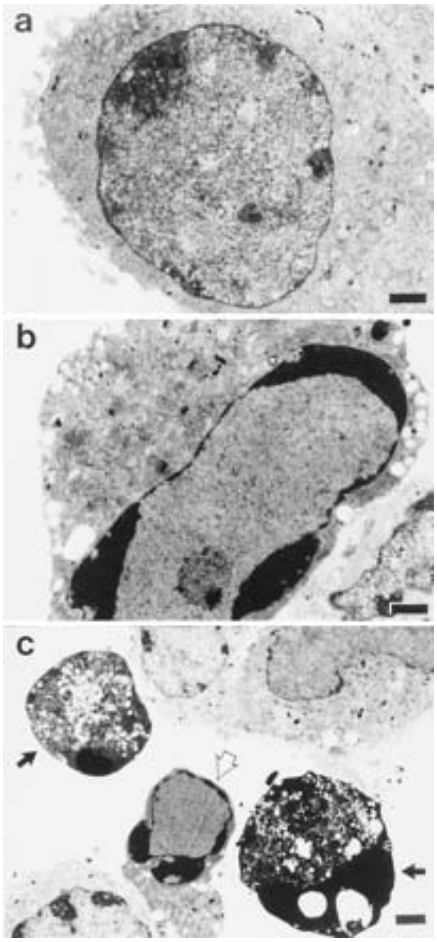

Fig. 6. Prolonged MPA treatment induced apoptotic changes in HIT-T15 cells. After treatment with 3 μg/ml MPA for 48 hrs, HIT cells cultured on glass coverslips were fixed and examined by electron microscopy. a, Control; b and c, treated with 3 μg/ml MPA for 2 days. In c, one cell (*open arrow*) was at the early stage of apoptosis, and two cells (*solid arrow*) were at the later stage of apoptosis. *Bars* in a and b = 1 μm; *bar* in c = 2 μm. This figure is adapted from the paper of Li et al. (1998) (***Endocrinology, Vol. 139, No. 9, 3752-3762)***

1.6. Aim of this study

IMPDH is a rate-limiting enzyme for the synthesis of GTP, dGTP and GNs (Jayaram et al., 1999; Metz and Kowluru, 1999). Alterations in the expression or activity of IMPDH affect cell proliferation, differentiation and apoptosis (Catapano et al., 1995; Li et al., 1998; Jayaram et al., 1999; Cohn et al., 1999; Metz et al., 2001). These effects may be mediated directly by GTP or indirectly via GTP-binding proteins (Metz and Kowluru, 1999). Based on the previous work (Li et al., 1998), this study has focused on the following three topics to define the mechanism underlying the apoptosis of insulin-secreting cells induced by GTP depletion with MPA treatment.

1.6.1. Does tTG participate in MPA induced β-cell apoptosis?

As described above, tTG is a unique protein with dual functions, cross-linking of polypeptides and coupling of certain receptors to phospholipase C (Feng et al., 1996;

Feng et al., 1999). In recent years the results from some studies suggest tTG may be also involved in the initiation of apoptosis. Increases of tTG expression and/or activity of tTG were observed during apoptotic cell death under various circumstances (Autuori et al., 1998). It is known that tTG activity is regulated by the levels of both Ca^{2+} and GTP in the cells; an increase of the former or a reduction in the latter stimulates tTG activity (Melino et al., 1998). Moreover, earlier work found that MPA specifically caused dramatic decreases of GTP level (by 71-74%) and GTP/GDP ratio (by 43-46%) after 18-h treatment in HIT cells (Meredith, 1997). Therefore, it is likely that tTG activity in β-cells would be elevated by GTP depletion due to MPA.

In the light of the above observations, this work aimed at evaluating the possible involvment of tTG in MPA-induced apoptotic death in HIT cells. We tried to examine the change of tTG activity after treatment of cells with different concentrations of MPA for various time periods, in order to find the possible relationship between tTG activity and MPA-induced apoptosis. In addition, the possible role of tTG in apoptosis due to GTP depletion was also examined by using tTG inhibitors (such as monodansylcadaverine and putrescine, or by lowering free Ca^{2+} concentrations in culture medium) to potentially block the cell death.

1.6.2. Is any caspase involved in MPA induced β-cell apoptosis?

Apoptosis may or may not involve caspases. In most cases, however, activation of caspases plays a critical role as either initiators or effectors in the execution of apoptotic cell death (Kumar, 1999; Earnshaw et al., 1999). Since it was unclear whether any caspase is implicated in GTP-depletion induced apoptosis of insulin-secreting cells, a goal of this study was to address this issue by measuring the activity of caspase-1 through caspase-10 after MPA treatment for various periods to identify the potential initiative and executive caspases. Furthermore, the role of caspases in

GTP-depletion induced cell death was also investigated by using selective caspase inhibitors.

1.6.3. Is any cell cycle regulator(s) important for MPA-induced β-cell apoptosis?

Apoptosis may occur at any stages of the cell cycle. However, the transition from G1 to S phase is regarded as a crucial point for deciding the cell growth and apoptosis and, in particular, cell progression from late G1 into S phase is regulated in many cells by p53 and CDKs (El Deiry et al., 1993; Roberts et al., 1994; Meikrantz and Schlegel, 1995). Several studies have also suggested the importance of cell cycle molecules in islet β-cell growth and death and in the induction of diabetes (Zhang et al., 1999b; Kaneto et al., 1999a; Rane et al., 1999; Tsutsui et al., 1999).

MPA depletes cellular GNs, which causes partial reduction in RNA synthesis and drastic inhibition of DNA synthesis, resulting in growth arrest and induction of apoptosis (Li et al., 1998; Cohn et al., 1999; Metz and Kowluru, 1999; Allison and Eugui, 2000; Yalowitz and Jayaram, 2000). However, little is known about the relationship between inhibition of mitogenesis and induction of apoptotic death due to GN depletion in insulin-secreting cells. It has been reported that MPA inhibited cell proliferation by specifically affecting the cells in S phase (Cohen et al., 1981; Nguyen et al., 1984; Knight et al., 1993; Cohn et al., 1999), while other studies indicated that an inhibition of *de novo* GNs synthesis blocked the transition of cell cycle from G1 to S phase (Laliberte et al., 1998). Interestingly, p53 may interact with IMPDH since the former down-regulated IMPDH activity and its protein and mRNA levels (Sherley, 1991; Liu et al., 1998b; Liu et al., 1998a; Yalowitz and Jayaram, 2000). Accordingly, it is possible (and previously unexplored) that the expression of some cell-cycle regulating proteins may be changed and implicated in β-cell apoptosis triggered by GTP depletion. In this study, we examined the levels of three important cell-cycle

regulators (p53, $p27^{KIP1}$ and $p21^{WAF1/CIP1}$) in the course of induction of apoptosis of HIT-T15 cells by GTP depletion, in order to investigate their role in this scenario.

Chapter 2

Materials and Methods

2.1. Materials

General laboratory chemicals and solvents were of reagent grade. Special reagents and chemicals used in this study are listed below in detail according to their sources:

BIOMOL, Plymouth Meeting, PA, USA

N-acetyl-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Tyr-Val-Ala-Asp-aldehyde, YVAD-CHO, a specific inhibitor for caspase-1

Boehringer Mannheim GmbH, Mannheim, Germany

Ac-leu-leu-argininal·½ H_2SO_4 (leupeptin)

N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES)

Sheep polyclonal anti-p53 (wild and mutant) antibody conjugated with peroxidase, anti-p53-protein-POD (polyclonal BMG-1B1)

Bio-Rad Laboratories, Hercules, CA, USA

Acetyl- Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC), a specific substrate for caspase-3, -7 and -10

Acetyl- Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin (Ac-LEHD-AFC), a specific substrate for caspase-9

Acetyl- Leu-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin (Ac-LETD-AFC), a specific substrate for caspase-8

Acetyl-Trp-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin (Ac-WEHD-AFC), a specific substrate for caspase-1, -4 and -5

Acetyl-Val-Asp-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin (Ac-VDVAD-AFC), a specific substrate for caspase-2

Acetyl-Val-Glu-Ile-Asp-7-amino-4-trifluoromethyl coumarin (Ac-VEID-AFC), a specific substrate for caspase-6

N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK), a general inhibitor for caspases

N-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone (Z-VDVAD-FMK), a specific inhibitor for caspase-2

Bio-Rad Protein Assay Dye Reagent Concentrate and protein standard

Coomassie Brilliant Blue R-250 (protein assay dye reagent)

Immun-blot polyvinylidine difluoride (PVDF) (0.45 μm) membrane

Kaleidoscope prestained standards

Nitrocellulose membrane (0.45 μm)

Stock solution for eletrophoresis, 30% Acrylamide/N, N'-methylenbisacrylamide (total monomer to crosslinker ratio at 37.5:1)

Biosource International, Nivelles, Belgium

Interleukin-1β, human recombinant protein

Calbiochem, La Jolla, CA

Caspase-3 inhibitor (DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-aldehyde)

E. Merck, Germany

β-Mercaptoethanol

Dimethyl sulphoxide (DMSO)

Formamide

Paraformaldehyde

Fisher Scientific, Leicestershire, UK

N-butanol

Tween-20

Gibco-Invitrogen Corporation, NK, USA

Bovine serum albumin (BSA)

Fetal calf serum (FCS)

New born calf serum (NBCS)

Protein A agarose

J. T. Baker, Phillipsburg, NJ, USA

Tris hydroxymethyl-aminomethane (Tris)

NeoMakers,Fremont, CA, USA

Tubulin-β Ab-4, positive loading control for Western blotting

ICN Biomedicals Inc, Aurora, OH, USA

3-(3-cholamidopropyl) dimethylammonio-1-propane sulfonate (CHAPS)

PhamMingen, San Diego, CA;

Polyclonal rabbit anti-caspase-3 (CPP-32, YAMA, APOPAIN) antibody

Purified mouse anti-cytochrome *c* monoclonal antibody (7H8.2C12, mouse IgG_{2b})

Purified mouse anti-human caspase-2 (ICH-1) monoclonal antibody

Pharmacia Biotech AB, Sweden

Ammonium persulphate (ASP)

Bromophenol blue

Tetramethylethylenediamine (TEMED)

Pierce, Rockford, IL, USA

5-(biotinamido) pentylamine, horseradish peroxidase (HRP)-conjugated streptavidin

Anti-Goat IgG, (H+L), peroxidase conjugated

Anti-rabbit IgG peroxidase conjugate

EZ-link 5-(biotinamido) pentylamine

Streptavidin, horseradish peroxidase conjugated

Supersignal West Dura; extended duration substrate

Supersignal West Pico; chemiluminescent substrate

Western blotting stripping buffer

Promega Corpo*rat*ion, Madison, WI, USA

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)

Santa Cruz Biotechnology, Santa Cruz, CA, USA

Affinity-purified polyclonal antibody (from goat) of $p21^{WAF1/CIP1}$ (c-19)

Affinity-purified polyclonal antibody (from goat) of $p27^{KIP1}$ (c-19)

Anti-goat Ig G-HRP conjugated

Anti-mouse IgG-HRP conjugated

Anti-rabbit IgG-HRP conjugate

Anti-RAIDD, rabbit polyclonal IgG

RAIDD (full length), Western blotting positive control

Sigma Chemical Co., St. Louis, MO, USA

Adenosine

Aprotinin

Bisbenzimidazole dye Hoechst 33258 (H33258)

Diethylpyrocarbonate (DEPC)

Dithiothreitol (DTT)

DNA standard (sodium salt, from calf thymus)

Ethidium bromide (EB)

Ethylene Glycol-bis (β-Aminoethyl ether)-N, N, N'N'-Tetraacetic acid (EGTA)

Ethylenediaminetetraacetic acid (EDTA)

Guanosine

Interleukin-1-β, rat recombinant

LDH assay kits (Cat. No. 228-100P)

Leupeptin, trifluroacetate salt

L-mimosine

Monodansylcadaverine (MDC)

Mouse monoclonal anti-β-tubulin

Mycophenolic acid (MPA)

N- [2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES)

o-phenylenediamine dihydrochloride

Penicillin

Pepstatin A

Phenazine methosulfate (PMS)

Phenylmethylsulfonylfluoride (PMSF)

Putrescine dihydrochloride

Ribonuclease A

RPMI 1640 medium, with L-glutamine and without sodium bicarbonate

Sodium dodecyl sulphate (SDS)

Streptomycin

Triton X-100

Upstate Biotechnology, Lake Placid, NY, USA

Anti-type II transglutaminase (goat polyclonal IgG)

2.2. Methods

2.2.1. Cell culture and storage

Cell culture

In this study, insulin-secreting HIT-T15 cells (passage 76-83) were used. The cells were maintained in RPMI 1640 supplemented with 10% decomplemented fetal calf serum (v/v), 100 i.u. penicillin/ml and 100 μg streptomycin/ml in a 5% CO_2 incubator at 37^{o}C. The cells were harvested upon reaching confluence, and passed weekly by

trypsinization and seeding at a density of 1.0 x 10^6 /ml in culture dishes. The culture medium was changed every 48 hrs.

During passing, the cells were rinsed with 37^{o}C phosphate-buffered saline (PBS) and incubated in 0.025% trypsin [5:45:50 of 0.5% trypsin stock: PBS (Ca/Mg-free): EDTA (0.54 mM)] for 5-8 min to detachment. Cold RPMI 1640 with 10% new born calf serum (NBCS) was added to terminate trypsinization. The resulting cell suspension was centrifuged at 130 x *g* at room temperature for 5 min. After discarding the supernatant, the cells were resuspended in RPMI 1640 with 10% fetal calf serum plus antibiotics and ready for seeding.

For experiments, cells were seeded in dishes or multi-well plates. When cells had approached more than 80% confluence, test agents were added to the medium for treatment of various time periods.

Cell storage

After washing the trypsinised cells with culture medium, they were resuspended in 1 ml of freezing medium (RPMI 1640 containing 20% fetal calf serum, 10% DMSO) and pipetted into cryo-tubes (around 1x10^6 cells). The tubes were packed with a cotton pad in a polystyrene box and placed at 4^{o}C for half an hour. The box was then left at –70^{o}C overnight, and the cryo-tubes finally transferred into a liquid nitrogen tank for long term storage.

2.2.2. Detection of apoptosis by MTS test and DNA content determination

Sustained (24-48 hrs) treatment of HIT cells with MPA caused apoptosis (Li et al., 1998). MPA stock solution (2 mg/ml) was prepared in ethanol and added into culture medium directly to reach final concentrations of 0.01-10 μg/ml for up to 48 hrs.

MPA-induced apoptosis of HIT cells was evaluated by MTS test and DNA content (Li et al., 1998). Our previous studies have demonstrated that these assessments are good markers to monitor GN-depletion induced apoptotic cell death which had been formally established by more specific means such as electron microscopic examination and measurements of DNA-laddering (Li et al., 1998).

Cell metabolic viability was monitored by MTS test, which detects reduction of a tetrazolium salt into a colored and water-soluble formazan in intact cells (Cory et al., 1991; Segu et al., 1998; Li et al., 1998; Huo et al., 2002). Dehydrogenases in metabolically functional cells accomplish the conversion of MTS into the formazan using PMS as the electron-coupling reagent (Cory et al., 1991). HIT cells cultured on 96-well plates were treated with test agents for various periods of time. After one gentle wash to discard culture medium and any floating cells, a mixture of MTS and PMS in fresh RPMI 1640 was added in the wells and the plates were incubated for 30 min. The absorbance of the formazan at 490 nm was measured in 96-well plates using a plate reader. We have confirmed that the intensity of absorbance from formazan product is proportional to the number of control living cells added into the wells.

For assessing cell death by determination of DNA content, cells seeded in microtiter plates were washed to remove culture medium and any floating cells. The attached cells were lyzed by sonication in distilled water. Aliquots of the cell lysates were reacted with the fluorescent DNA-binding dye, Hoechst 33258 (Rago et al., 1990), in 96-well plates. The fluorescence was measured by a fluorescent plate reader (SPECTRAmax^{TM} GEMINI, from Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 356 nm and 448 nm, respectively. DNA contents in the wells were calculated by comparison with the standard curves.

The raw values from MTS test and DNA measurements were affected by many factors such as cell numbers, probe concentrations and equipment function. Indeed, there is variance in the cell numbers used between experiments, as well as between time points for controls due to proliferation and for treated cells due to cell loss. As a consequence, it is difficult to compare the raw data between individual experiments (including the different time points of treatment within one experiment) and to pool the data for statistical analyses. Therefore, we always measured the samples of both treated cells and control cells at any time point (they were seeded at the same time in triplicate each) and calculated the mean values for each condition. Afterwards, the ratio of mean values of treated samples was expressed as percentage of control. In this way, the change due to a given treatment (dose or time) could be assessed more precisely.

2.2.3. Flow cytometric analysis for DNA fragmentation and cell cycle

Cell cycle were determined by propidium iodide (PI) staining and flow cytometry as described by Nicoletti *et al* (Nicoletti et al., 1991). This assay is also able to measure the fragmented nuclei and thus detect the subdiploidy apoptosis (Nicoletti et al., 1991). PI is the most widely used dye for the analysis of DNA by flow cytometry. It stains all double-stranded regions of both DNA and RNA by intercalating between the stacked bases of the double helix. The dye can not penetrate an intact cell membrane and, therefore, permeabilization such as ethanol fixation is necessary.

In this study, HIT cells were cultured in 6-well plates with 3 μg/ml MPA for 8-48 hrs. After one gentle rinse by PBS, the cells were collected by trypsinization and centrifugation. Then the cell pellets were gently resuspended thoroughly in 0.5 ml PBS followed by 2-h fixation (at 4^{o}C) in 4.5 ml 70% ethanol. Cells suspended in 70% ethanol were stored at -20^{o}C if PI staining was not performed immediately. For PI

staining, the ethanol suspended cells were centrifuged for 5 min at 200 x *g* to remove cell debris. The cell pellets were re-suspended in 5 ml PBS and centrifuged for 5 min at 200 x *g*. Afterward, the cells pellets were incubated in 1 ml PI/Triton X-100 staining solution (containing 20 μg/ml PI, 0.1% Triton X-100 and 0.2 mg/ml RNAse A in PBS) for 30 min at room temperature. Flow cytometric analysis was carried out using a fluorescence-activated-cell-sorter (EPICS Elite ESP, Beckman Coulter, Hialeah, FL). An excitation source of 488 nm was achieved using a 15 mW air-cooled argon-ion laser. Fluorescence emission was collected through a 610 nm band pass filter for PI. PI fluorescence data were collected on a linear scale. Ten-thousand cells were evaluated for each sample. The flow rate was kept at 200-300 cells/sec. The data were processed and analyzed using WinMDI software (Scripps Institute, La Jolla, CA).

The number of floating and rinsed cells not included in the flow cytometric analysis and other two apoptotic assays (MTS test and DNA measurements) was varied depending on the time periods of MPA treatments (5.7%, 7.3%, 8.3%, 9.3%, 12.8%, 19% and 33.9% at 0, 8, 16, 24, 32, 40 and 48 hrs, respectively; n=3). Almost all of these cells (>94%) were dead cells or apoptotic cells as assessed by PI staining and flow cytometry.

2.2.4. Protein concentration assay

Protein contents were measured by Bio-Rad assay based on the change of absorption of Coomassie brilliant blue G-250 dye (shifts from 465 nm to 595 nm when binding to protein) in response to various concentrations of protein. The dye binds primarily to basic (especially arginine) and aromatic amino acid residues. The assay is useful for measuring proteins and polypeptides with MW greater than 3,000-5,000, depending on the charged groups.

Aliquots of samples or protein standard (181.25-1,450 μg/ml) were mixed with the reagent in 96-well plates and incubated at room temperature for 10 min. The absorbance at 595 nm was measured with a microplate reader. The protein concentrations in samples were calculated by comparing the standard curve obtained from known concentrations of protein which was run in parallel.

2.2.5. Assay of *in situ* tTG activity

In situ tTG activity was measured in cell extracts following the procedures by Zhang *et al*, in which a biotinylated tTG substrate was loaded into living cells and the generated product was measured by a streptavidin-linked enzyme assay (Zhang et al., 1998).

HIT-T15 cells were cultured in 24-well plates and treated with different concentrations of MPA alone or combined with other test agents for 24 or 48 hrs. On the day of the experiment, cells were pre-incubated in 250 μl Krebs-Ringer-bicarbonate-Hepes (KRBH) buffer (supplemented with 0.1% BSA and 10 mM glucose) containing 2 mM of the tTG substrate, 5-(biotinamido) pentylamine, for 1 hr. After one wash with cold PBS, 0.2 ml homogenizing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml each of aprotinin, leupeptin, and pepstatin) was added to each well and cells were subjected to sonication (3 x 10 sec) on ice. Protein contents were determined by Bio-Rad assay. Subsequently, 10 μg of homogenate protein was diluted to 50 μl with coating buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA) and added to the 96-well microtiter plates. After incubation overnight at 4^{o}C, 200 μl of blocking buffer [100 mM boric acid, 20 mM sodium borate, 80 mM NaCl (BBS) containing 5% BSA, 0.01% Tween-20, and 0.01 % SDS] was added to each well. The plates were incubated for 2 hrs at 37^{o}C. After one wash with a rinse buffer (BBS supplemented

with 1% BSA and 0.01% Tween-20), 100 μl of horseradish peroxidase (HRP)-conjugated streptavidin (1:2000) in BBS with 1% BSA and 0.01% Tween-20 was added and the plates were left at room temperature for 1 hr. After 4 rinses, 200 μl of substrate solution (0.04% o-phenylenediamine dihydrochloride in 50 mM sodium citrate phosphate buffer, pH 5.0) was added to each well. The plates were incubated at room temperature for 15 min and the reaction was terminated by adding 50 μl of 3 M HCl. Optical density of HRP-generated product was measured with a plate reader at 492 nm. tTG activity was expressed as the percentage of basal activity in control samples.

2.2.6. Caspase activity measurement

Measurements of caspase activity are simplified by using fluorogenic synthetic oligopeptide substrates (Komoriya et al., 2000). These amino acid sequences are identical or similar to those found in full-length protein substrates, and where differences occur, the optimized synthetic substrates appear to be cleaved as efficiently or better than the full-length proteins. Substrates are synthesized by modifying the caspase cleavage site (C-terminal aspartic acid) with 7-amino-4-trifluoromethyl coumarin (AFC). When liberated from the peptide, AFC produces an optical change resulting in emissions of fluorescence.

HIT cells were cultured in 6-well plates. For each independent experiment, duplicates of both control and treated wells were applied for any time point of treatment. Activity of individual caspase in samples from control and treated cells at any time point of treatment was measured and compared. The results were expressed as percentage of control. After decanting medium and rinsing cells with cold PBS, 100 μl lysis buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 350 μg/ml PMSF, 10 μg/ml pepstatin A, 10 μg/ml aprotinin and 20 μg/ml leupeptin) was added to each

well and cells were scraped with a cell lifter and transferred to clean tubes. The tubes were frozen and thawed for 4 cycles to lyze the cells by transferring from a -20^{o}C freezer to a 37^{o}C water bath. Lysates were centrifuged (Eppendorf) at 4^{o}C for 30 min at full speed. The supernatants were transferred to clean tubes and kept on ice if the assay was to be performed within 1 hr or, otherwise, stored at -70^{o}C. The caspase activity assay was performed according to the protocol provided by the supplier. In brief, the reaction components containing caspase substrates were thoroughly mixed with a blank or test samples in a 96-well microtiter plate. After incubation at 37^{o}C for 3 hrs, the plates were scanned by a fluorescent plate reader to measure the AFC fluorescence at excitation and emission wavelengths of 395 nm and 525 nm, respectively. The increase in AFC fluorescence signal was proportional to the enzyme activity. All values were corrected by blank readings and the enzyme activity was normalized to a fixed protein concentration.

2.2.7. Isolation of mitochondria-enriched and cytosolic fractions

All following steps were performed at 4^{o}C as described previously (Li et al., 1996). After washing with cold PBS, HIT cells were detached by scraping and suspended in homogenization buffer containing20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 250 mM sucrose, 1 mM DTT and the following protease inhibitors (in μg/ml): 10 leupeptin, 4 aprotinin, 2 pepstatin and 100 PMSF. The cells were first subjected to cavitation in a nitrogen bomb (from Parr) under 10 bars for 30 min and then the achieved cell suspensions were further homogenized by using a Dunce tissue grinder (from Kontes) with the tight pestle. The homogenates were centrifuged at 900 x *g* for 10 min to remove unbroken cells and nuclei. The supernatants were then centrifuged at 5,500 x *g* for 20 min. The pellets (rich in mitochondria) were resuspended in homogenization buffer and sonicated for 3 times (5 sec each). Cytosol was achieved

by centrifuging the post-mitochondrial supernatant at 100,000 x *g* for 60 min. Protein concentrations were determined and samples were aliquoted and kept at -70^{o}C.

2.2.8. Examination of cell morphology

HIT cells were cultured on chambered coverglass (Nalge Nuce, Naperville, IL) with test agents for indicated time periods. Non-adherent cells were removed by changing medium. Morphology of living cells was examined under differential interference contrast (DIC) by a Curl Zeiss confocal microscope (objective: Plan-Neofluar, 20x/0.5).

2.2.9. Immunoprecipitation of RAIDD and caspase-2

HIT cells cultured in 6-well plates were treated with MPA (3 μg/ml) for indicated time periods. All procedures below were performed at 4^{o}C. After washing in cold PBS buffer, the cells were lysed in cold immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 10 mM Tris pH 7.4, 1 mM EGTA pH 8.0, 0.2 mM sodium ortho-vanadate, 0.2 mM PMSF, 0.5% NP-40) for 30 min. Cell homogenates scraped from the plates were then passed 10 times through a 27 gauge needle to disperse any large aggregates, and centrifuged (16,000g) for 15 min to get whole cell lysates. For initiation of immunoprecipitation, 2 μg (10 μl) anti-RAIDD antibody was added into each 1.5-ml Eppendorf tube containing 1 ml whole cell lysate (total 100 μg protein) and incubated in constant agitation for 1 hr. Afterwards, 10 μl of 50% agarose-immobilized Protein A was added to the mixture and incubated in agitation for 30 min. After centrifuging for 4 min (16,000 x *g*), the pellets were rinsed by cold immunoprecipitation buffer for 3 times and resuspended in 30 μl double-concentrated electrophoresis sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol). The samples were boiled and subjected to

electrophoresis and Western blotting for detection of RAIDD and caspase-2 as described below.

2.2.10. Western blotting of tTG, caspase-2, RAIDD, cytochrome *c*, p53, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$

Cells cultured in dishes or multiwell plates were washed with cold PBS, scraped in a homogenizing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin) by cell-lifers, and sonicated on ice 3 times (10 sec each). The samples were stored at -70^{o}C if not immediately used for Western-blotting.

Some of experimental conditions for detecting tTG, caspase-2, RAIDD, p53, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ in cell homogenates by Western blotting were summarized in Table 3. Aliquots of samples were boiled in a loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 20% glycerol, 4% SDS, and 0.2% bromophenol blue) for 5 min and centrifuged at 9,000 x *g* for 5 min. These samples or aforementioned immunoprecipitation samples were subjected to a 10% SDS-polyacrylamide gel for electrophoresis (SDS-PAGE) and proteins were then electro-transferred to nitrocellulose or polyvinylidine difluoride (PVDF) membranes. The membranes were blocked in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.2% Tween-20) containing 5% dried non-fat milk (using BSA for p53) for 1 hr at room temperature. Subsequently, membranes were blotted with the antibodies (see Table 3 for the details of dilution) against tTG, caspase-2, RAIDD, p53, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ for 1-2 hrs at room temperature. After three washes in TBST (5 min each), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-goat IgG, anti-rabbit IgG or anti-mouse IgG as appropriate) for 60 min

Table 3. Selected conditions for Western blotting of different proteins in this study

Protein	Sample amount (μg protein)	SDS-PAGE (%)	Blocking buffer/Time (h)	1st Ab conc.
tTG	20	10	5% dried non-fat milk/1	1:1000
Caspase-2,	20	10	5% dried non-fat milk/2	1:250
RAIDD,	20	10	5% dried non-fat milk/1	1:1000
Cytochrome *c*	20, mitochondria 40, cytosol	15	5% dried non-fat milk/2	1:1000
p53,	20	10	5% BSA/2	1:500
$p21^{WAF1/CIP1}$	20	10	5% dried non-fat milk/2	1:500
$p27^{KIP1}$	20	10	5% dried non-fat milk/1	1:1000

at room temperature. The membranes were washed again (3 x 15 min) in TBST. The light-emitting signals were detected using the enhanced chemiluminescence (ECL) system followed by exposure to an x-ray film. The films were scanned by desktop scanner and data saved as Tagged-Image File Format (TIFF) files. The optical density of bands on the films was semi-quantified by computer-assisted densitometry.

For Western blotting of cytochrome *c*, aliquots of cytosol (40 μg protein) and mitochondrial lysate (20 μg protein) were subjected to 15% SDS-PAGE, and proteins then transferred to PVDF membranes. The membranes were blocked with dried non-fat milk and subsequently blotted with cytochrome *c* antibody. Afterwards, the immuno-detection was performed as described above. In this study, levels of β-tubulin were served as loading control of Western blotting.

2.2.11. Determination of lactate dehydrogenase (LDH) release

Cells (0.15×10^6) were seeded in 96-well plates and cultured in normal medium for 2 days and then in serum-free culture medium with test agents for 24 or 48 hrs. Fetal calf serum was excluded during the treatment periods since the medium containing serum exhibited unknown factors which increased the blank signal dramatically (equivalent to 3 times of LDH activity in HIT cell homogenates) and made it impossible to meaningfully measure the activity of released LDH in our samples. At

the end of treatment, equal amounts of supernatant from each well were mixed with the LDH assay solution (kits from Sigma, St. Louis, MO) and incubated for 30 min at 37^{o}C. The absorbance at 340 nm was measured and the LDH activity was calculated according to the instructions provided the kit supplier.

2.2.12. Statistical analysis

All data are presented as the means ± SE of the number of independent experiments. Statistical analysis of the data was performed by the Student's *t* test or one-way analysis of variance (ANOVA) followed by an appropriate *post hoc* comparison. The outcome of $p<0.05$ was considered statistically significant difference.

Chapter 3

Results

3.1. Role of tTG in GTP depletion induced apoptosis of insulin-secreting cells

3.1.1. Increase of *in situ* tTG activity and induction of apoptosis by MPA in HIT cells

As demonstrated previously (Li et al., 1998; Huo et al., 2002), sustained depletion of GTP with MPA for 24 or 48 hrs induced apoptotic death of HIT cells as assessed by MTS test (Fig. 7). This convenient assay is widely used for determining the number of viable cells (Buttke et al., 1993; Wong et al., 2001). Our previous studies indicate this test is a good way to monitor apoptotic cell death once the presence of the latter has been formally established by more specific means such as EM and DNA-laddering (Li et al., 1998) and more recently by flow cytometry (Huo et al., 2002).

The *in situ* tTG activity was significantly increased in parallel under the same conditions (Fig. 8). This effect was time-dependent. At 10 μg /ml MPA, the enzyme

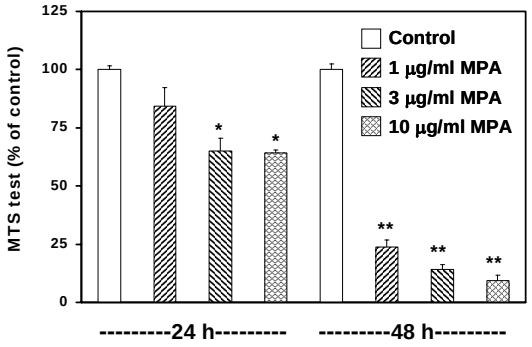

Fig. 7. Induction of apoptosis of HIT cells by MPA treatment in a dose-dependent manner. Cell seeded on 96-well plates were treated with various concentrations of MPA for 24 or 48 hrs. Cell viability was evaluated by MTS test (formazan production). Data are mean ± SEM of 4 independent experiments. * P<0.05 and ** P<0.01 *vs.* control.

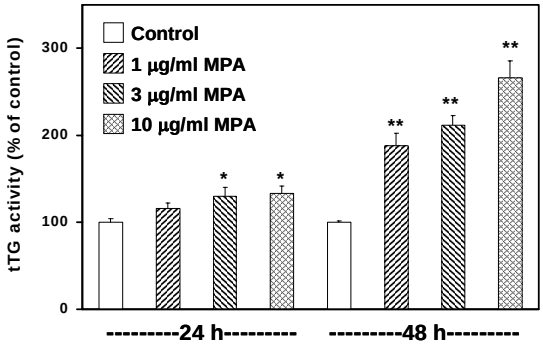

Figure 8. Increase of *in situ* tTG activity by GTP-depletion after MPA treatment in a dose-dependent manner. Cell seeded on 96-well plates were treated with various concentrations of MPA for 24 or 48 hrs. *In situ* tTG activity was measured by a streptavidin-linked enzyme assay detecting the formation of a tTG-dependent product from a loaded substrate. Values are mean ± SEM of 3 independent experiments. * P<0.05 and ** P<0.01 *vs.* control.

activity was increased by 33% after treatment of 24 hrs and increased further (+133%) after 48 hrs. The two effects of MPA (promotion of cell death and enhancement of tTG activity) were closely correlated in a dose-dependent manner (Fig. 9). Furthermore, data in Fig. 9 demonstrated that both actions were prevented by the provision of guanosine (which restores GTP content and GTP/GDP ratio) but not by adenosine (which restores any possible reduction in ATP content) (Metz et al., 1992; Meredith et al., 1997; Li et al., 2000), indicating that the enhanced tTG activity, similar to the induction of apoptosis, in MPA-treated cells is due specifically to the depletion of GNs.

The increased tTG activity during MPA treatment could result from elevated tTG mass and/or stimulation of enzyme activity. To address this question, we evaluated tTG protein levels by Western blotting. Surprisingly, MPA treatment did not enhance tTG mass but rather moderately decreased its levels at higher concentrations (Fig. 10), suggesting that GTP-depletion may have a negative effect on tTG protein synthesis

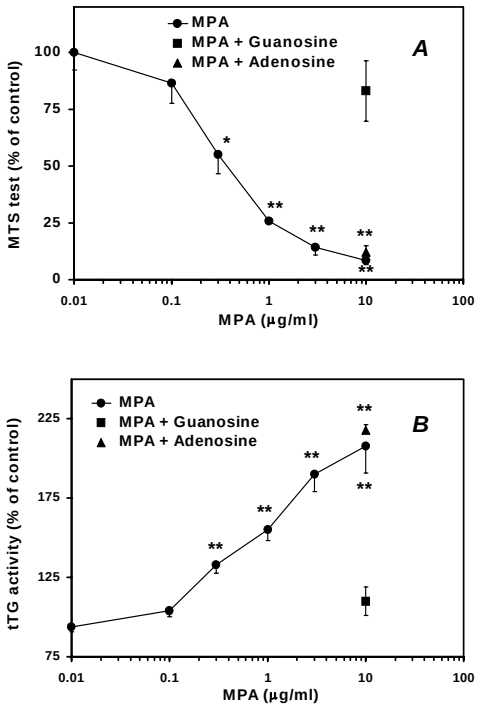

Fig. 9. Prevention of MPA-promoted increase of *in situ* tTG activity (*A*) and induction of cell death (*B*) by the provision of guanosine but not adenosine. HIT cells in 96-well plates were cultured with a series concentrations of MPA for 48 hrs. Guanosine (500 µM) or adenosine (500 µM) was also included upon appropriate. Cell viability was evaluated by MTS test (formazan production) and *in situ* tTG activity was measured by the formation of product from a loaded substrate for the enzyme. Data are mean ± SE of 4 independent experiments. * P<0.05 and ** P<0.01 *vs.* control.

and/or degradation. Neither tTG degradation products nor its polymers could be detected in our studies (data not shown). The decrease of tTG mass was not observed at an early time point (24-h) but was visible after 48-h MPA treatment. Possibly, MPA might induce tTG secretion from HIT cells. This MPA-induced alteration in tTG mass was also blocked by addition of guanosine but not adenosine (Fig. 10). Thus the increase of tTG activity in GTP-depleted β-cells was due to the activation of the enzyme *per se* rather than an increase of its mass.

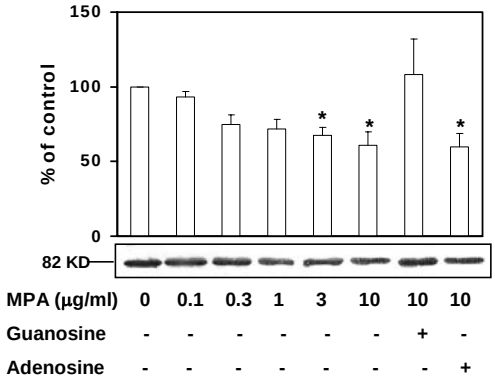

Fig. 10. Decrease of tTG expression at protein level after 48-h MPA treatment. tTG was determined by Western-blotting followed by densitometry analysis. The effect of MPA on decrement of tTG mass was blocked by inclusion of guanosine (500 μM) but not adenosine (500 μM). The blotting is the representative of 4 independent experiments under the same conditions. Bar values are mean ± SE from density analysis of these experiments. * $P<0.05$ *vs.* control.

3.1.2. Effects of tTG inhibitors on tTG activity and apoptosis during MPA treatment

The relationship between increased tTG activity and apoptotic cell death during GTP-depletion by MAP was studied under condition of tTG inhibition. In one series of experiments, two tTG inhibitors, monodansylcadaverine (MDC) or putrescine (Tsai et al., 1998), were co-cultured together with 3 μg/ml MPA for 48 hrs. The inhibitors had no apparent effect on cell viability and proliferation under basal conditions by themselves at the doses used in our study. In contrast, either 100 μM MDC or 6.25 mM putrescine was able to block the *in situ* tTG activity by almost half (42-43%) (Fig. 11A). However, they did not rescue the cells from apoptosis under the same conditions (Fig. 11B). In addition, the tTG inhibitors did not alter tTG mass during MPA treatment as assessed by Western-blotting (data not shown).

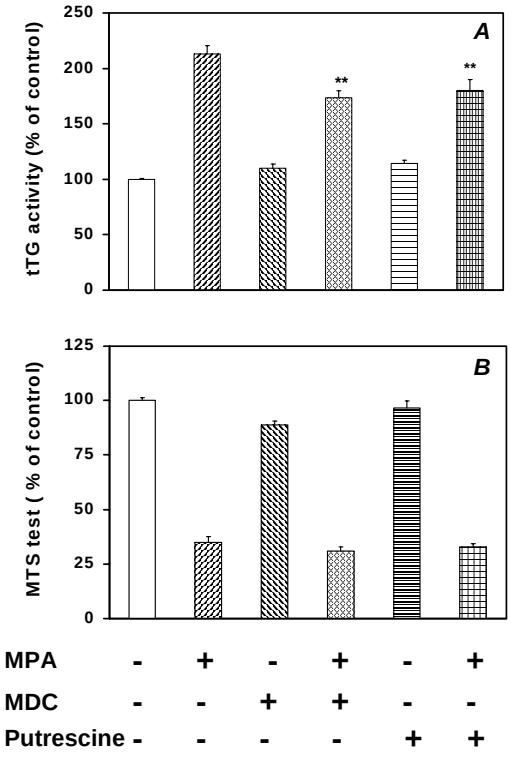

Fig. 11. Partial suppression of MPA-enhanced tTG activity (*A*) but failure of prevention of MPA-induced apoptosis (*B*) by two tTG inhibitors. HIT cells were treated with 3 μg/ml MPA alone or combined with 100 μM MDC or 6.25 mM putrescine in culture media for 48 hrs. Cell viability was evaluated by MTS test (formazan production) and *in situ* tTG activity was measured by a streptavidin-linked enzyme assay. Data are mean ± SE of 6 independent experiments. ** P<0.01 *vs*. MPA treatment alone.

In another series of experiments, free Ca^{2+} levels in culture medium were lowered to 80-120 nM by using the Ca^{2+} chelator EGTA, since tTG activity is not only regulated by GTP (suppression) but is also stimulated by Ca^{2+} (Smethurst and Griffin, 1996; Zhang et al., 1998; Melino and Piacentini, 1998). The results revealed that lowering of extracellular free Ca^{2+} levels almost abolished (by 91%) the enhanced tTG activity due to MPA (Fig. 12A), but failed to prevent cell death triggered by GTP depletion (Fig. 12B). Lowing free Ca^{2+} levels to 80-120 nM itself did not damage cell viability.

In addition, MPA-induced apoptosis was also examined by flow cytometry which determines the subdiploidy apoptosis (sub-G1 phase) due to DNA fragmentation (Huo et al., 2002). As shown in Table 4, 53% cells were detected undergoing apoptotic death after MPA treatment (3 μg/ml for 48 hrs). Putrescine, MDC and lowing free Ca^{2+} did not affect cell viability by themselves. In agreement with the results from MTS test (Fig. 11B), neither putrescine nor MDC was able to rescue the cells from MPA-induced cell apoptosis (Table 4). However, lowing free Ca^{2+} was able to slightly

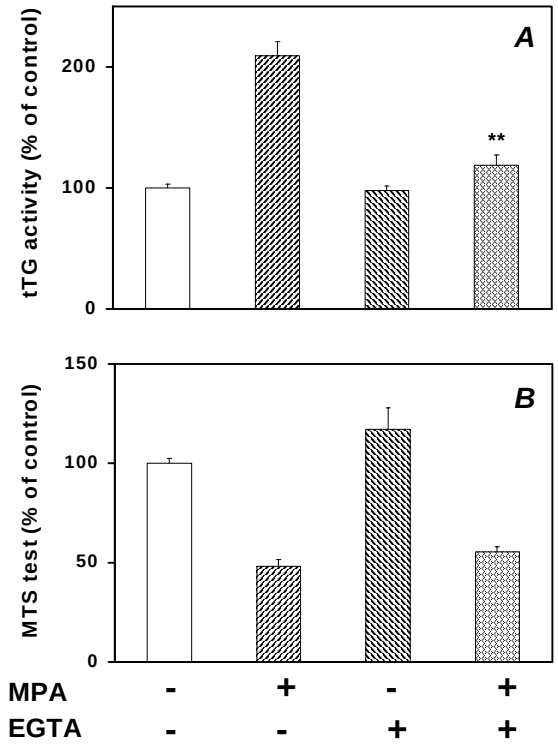

Fig. 12. Significant suppression of MPA-enhanced tTG activity by EGTA without prevention of MPA-induced apoptosis. HIT cells in 24-well plates were cultured with 3 μg/ml MPA for 48 hrs. The Ca^{2+} chelator EGTA (0.83 mM) was also included where appropriate. tTG activity (***A***) was measured by the formation a product from a loaded substrate for the enzyme and cell viability (***B***) was evaluated by MTS test. Values are mean ± SEM of 5 independent experiments. ** P<0.01 *vs.* MPA treatment alone

(by 29%) but significantly relieve MPA-evoked cell death. These data suggested that while tTG might have some small involvement, it appeared not to play a major role in the induction of apoptosis due to GTP-depletion.

Condition	Apoptotic cells (%)
Control	8.4 ± 1.3
MPA, 3 μg/ml	53.2 ± 2.7
Putrescine, 6.25 mM	11.4 ± 3.2
MDC, 100 μM	8.7 ± 2.6
Lowering free Ca^{2+}	10.7 ± 3.3
MPA + Putrescine	54.3 ± 6.1
MPA + MDC	51.3 ± 1.9
MPA + Lowering free Ca^{2+}	42.3 ± 1.8*
MPA + 100 μM Z-VAD-FMK	13.9 ± 2.0**
MPA + 200 μM Z-VAD-FMK	12.2 ± 1.7**

Table 4. Analysis of MPA-induced subdiploidy apoptosis by flow cytometry. HIT-cells in 6-well plates were cultured under the above conditions for 48 hrs. All cells were collected and stained by propidium iodide and subject to flow cytometric analysis as detailed in the Methods and Materials section. The cells in sub-G1 phase whose DNA were fragmented were counted as subdiploidy apoptosis**.** Values are mean ± SEM from 3-7 independent experiments. * p<0.05 and ** p<0.01 *vs*. MPA alone

3.1.3. Effects of caspase inhibitor on tTG activity in GTP depletion

It is well established that caspase activation is involved in both initiation and execution of apoptosis (Green and Reed, 1998; Budihardjo et al., 1999). To study

whether the increased tTG activity during GTP-depletion by MPA is related to caspase activation, a cell-permeable pan-caspase inhibitor, Z-VAD-FMK (Gamen et al., 1997; Bras et al., 1999), was employed. This peptide derivative is able to bind specifically to general caspases but the modification of its C-terminal aspartic acid with fluoromethyl ketone (FMK) renders it an irreversible caspase inhibitor rather a substrate (Talanian et al., 1997). At 100 μM, Z-VAD-FMK completely prevented apoptosis induced by GTP-depletion as assessed by MTS test (Fig. 13A) as well as the accompanying activation of several caspases (Huo et al., 2002). Similarly, MPA-induced apoptosis was prevented by the caspase inhibitor as determined by flow cytometry (Table 4). However, the same concentration, or an even higher one (200 μM), of the pan-caspase inhibitor did not prevent the increase of *in situ* tTG activity caused by MPA treatment (Fig. 13B). This suggested that the enhanced tTG activity occurs either up-stream or independent of caspase activation during MPA-treatment.

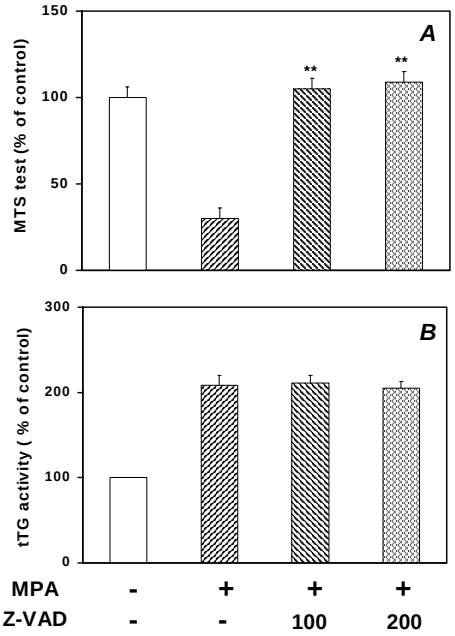

Fig. 13. Prevention by a pan-caspase inhibitor of apoptosis but not the increased tTG activity induced by MPA. HIT cells in multi-well plates were cultured with 3 μg/ml MPA for 48 hrs and the pan-caspase inhibitor Z-VAD-FMK (100 or 200 μM) was also included upon appropriate. Cell viability was evaluated by MTS test (***A***) and *in situ* tTG activity was measured by a streptavidin-linked enzyme assay (***B***). Data are mean ± SEM of 3 independent experiments. ** P<0.01 *vs.* MPA treatment alone.

3.1.4. tTG activity and cell morphology

Control HIT cells grew in clusters on culture plates and the cell boundary was ambiguous (Fig. 14A). MPA treatment caused the cells to round up and individual

cells became recognizable (Fig. 14B). In addition, many of these cells lost their normal brightness. Guanosine, which abolished MPA-induced tTG activation (Fig. 9), restored normal morphology (Fig. 14C) while adenosine had no such effect (Fig. 14D). However, the morphological changes induced by MPA treatment could only be marginally reversed by the pan-caspase inhibitor (Fig. 14E-F), allowing for a possible role of tTG in this process compatible with its proposed role in protein cross-

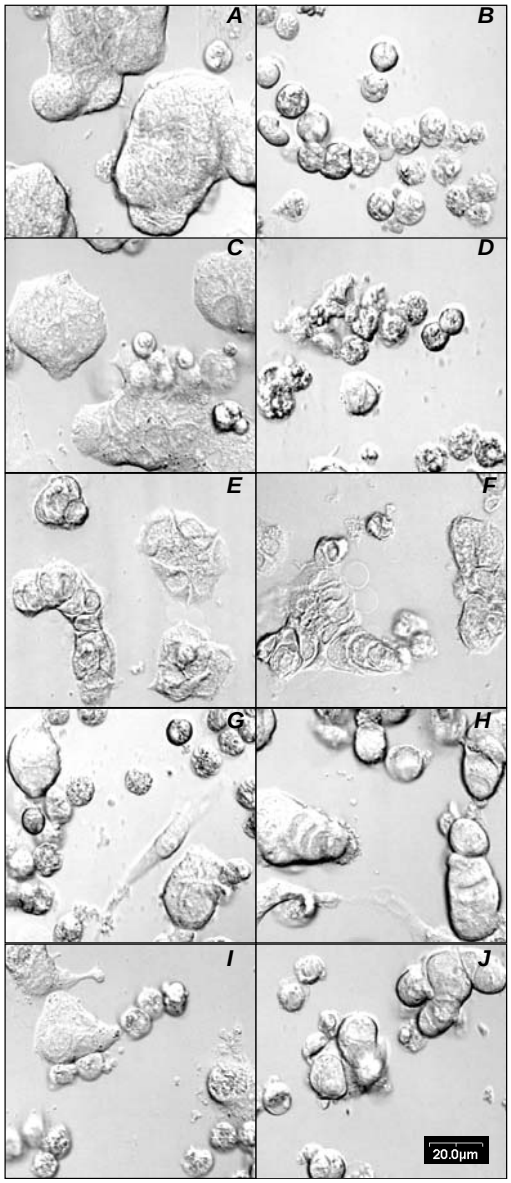

Fig. 14. Partial prevention of MPA-induced morphological alterations by tTG inhibitors. HIT cells in coverslip chambers were cultured for 48 hrs in the following conditions: control (*A*); 3 µg/ml MPA (*B*); MPA plus 500 µM guanosine (*C*); MPA plus 500 µM adenosine (*D*); MPA plus 100 µM (*E*) or 200 µM (*F*) Z-VAD-FMK; MPA plus 6.25 mM putrescine (*G*) or 100 µM MDC (*I*); MPA plus putrescine and 100 µM Z-VAD-FMK (*H*); and MPA plus putrescine and 100 µM Z-VAD-FMK (*J*). Cell morphology was examined under differential interference contrast (DIC) by microscopy. Photos are representative of 4 independent experiments

linking (Fesus and Thomazy, 1988; Greenberg et al., 1991; Fesus, 1998; Melino and Piacentini, 1998). Indeed, either putrescine or MDC was able to markedly prevent the morphological changes in MPA-treated cells (Fig. 14G, 14I) and this effect was more apparent in the presence of a caspase inhibitor (Fig. 14H, 14J); these observations matched the observation that they could only produce incomplete inhibition of MPA-induced tTG activation (cf. Fig. 11A).

It has been proposed that an effect of tTG is to stabilize dying cells by polymerization of substrate proteins (Fesus, 1998; Melino and Piacentini, 1998). Cross-linking of proteins by tTG might reduce the release of cytoplasmic materials from cells undergoing apoptosis and inhibition on tTG therefore might increase the release in apoptotic cells. In order to address this issue, we assessed LDH release from MPA-treated cells under various conditions (Fig. 15). There was no apparent release of LDH after 24-h treatment with MPA, whereas 48-h exposure to MPA caused an increase of LDH release by 191%. Neither a combination of two tTG inhibitors (MDC and putrescine) nor lowing Ca^{2+} by EGTA affected the MPA-induced LDH release. However, the pan-caspase inhibitor (Z-VAD-FMK) prevented the MPA-induced LDH release and also the increase in fragmented DNA measured by flow cytometry (cf. Fig.

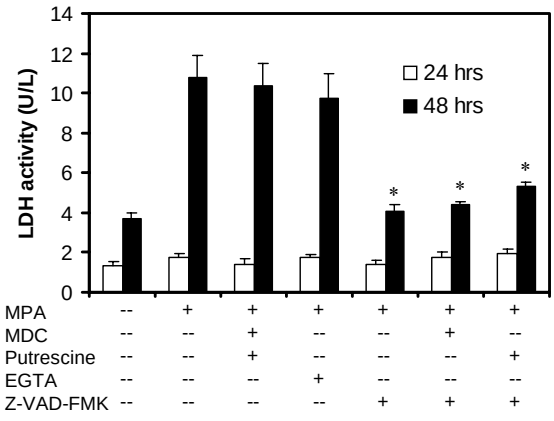

Fig. 15. LDH release from HIT cells after MPA treatment under various conditions. After HIT cells seeded in 96-well plates were treated with test agents in culture medium for 24 or 48 hrs, the LDH activity in the supernatants was measured as described in the Materials and Methods. Values are mean ± SEM of 5 independent experiments. * P<0.01 vs. MPA treatment alone. The final concentrations during treatment were: MPA, 3 mg/ml; MDC, 100 μ M; putrescine, 6.25 mM; EGTA, 0.83 mM; and Z-VAD-FMK, 100 μ M.

13A and Table 4). As expected, inclusion of tTG inhibitors did not significantly alter the reversing effect by Z-VAD-FMK on LDH leakage. These data support our conclusion that tTG does not play a major role in the phenomena associated with apoptosis, including leakage of cytoplasmic contents

3.1.5. Section summary

This part of work attempted to delineate the role of tTG in apoptosis of insulin-secreting HIT-T15 cells induced by GTP depletion with MPA. The results showed that GTP depletion elevated tTG activity. The time course and dose-response of this effect was in close relationship with apoptosis induction. The elevated enzyme activity was not due to an increase of its mass. Provision of guanosine completely prevented MPA-induced cell death and increase of tTG activity. Although tTG inhibitors and lowering of Ca^{2+} were able to suppress tTG activation, MPA-induced cell death was not or only slightly relieved under these conditions. A general caspase inhibitor was capable of preventing MPA induced apoptosis without affecting the tTG activation, indicating that GTP depletion can provoke the two events either independently or one after another. Importantly, the morphological changes accompanying apoptosis could be partially prevented by tTG inhibitors. These findings suggest that the effect of the marked increase in tTG activity during GTP-depletion induced apoptosis of insulin-secreting cells may be restricted to some terminal morphological alterations.

3.2. Importance of activation of caspases for the apoptosis induced by GTP-depletion in insulin-secreting cells

3.2.1. Induction of apoptosis by GTP depletion with MPA treatment

HIT cells were treated with 3 μg/ml MPA for up to 48 hrs. Flow cytometric analysis of PI-stained cells indicated that MPA induced cell arrest in G1 (diploid peak) phase (Fig. 16A and Table 5). The cells at S and G2/M phases were significantly reduced after MPA treatment at 16 h and beyond. Meanwhile, MPA increased the number of

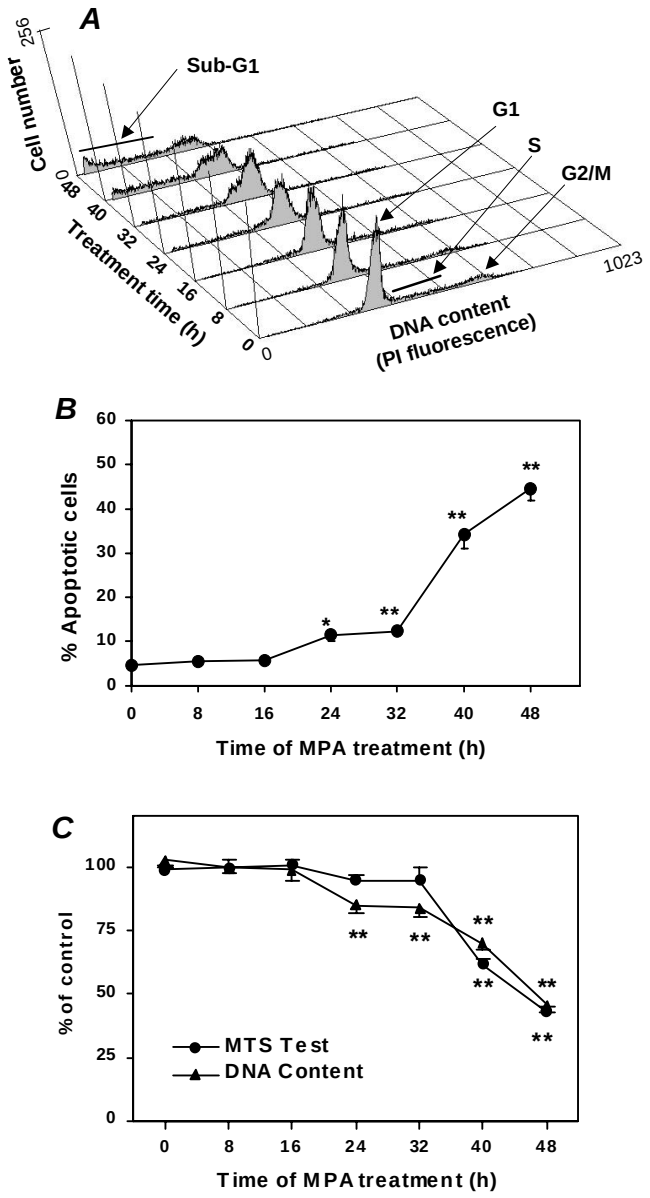

Figure 16. Time-course of cell death induced by MPA treatment. HIT cells seeded on multi-well plates were treated by MPA (3 μg/ml) in culture media for up to 48 hrs. (***A)***, Cells were stained with PI and subject to flow cytometric analysis of MPA effects on cell growth/death. The graph is a typical representative of 3 independent experiments. The cells whose DNA content was lower than G1 phase contained fragmented nuclei and were undergoing apoptosis. (***B)***, Percentages of MPA-induced subdiploidy apoptotic cells analyzed by flow cytometry. Values are mean ± SE from 3 independent experiments. (***C),*** Cell viability was evaluated both by formazan production in MTS test and by determination of DNA content in the wells under different conditions. Corrections were made for the influence of a slight increase (15-20% during 48 hrs) in the cell number of control wells in all data (both MTS test and DNA content) at indicated time points. Values are mean ± SE from 7 independent experiments. * $P<0.05$ and ** $P<0.01$ *vs.* zero time point.

cells with fragmented nuclei; the DNA content of these cells was lower than that at G1 phase (in sub-G1 fraction) (Fig. 16A and Table 5). These subdiploid cells with fragmented nuclei thus were undergoing apoptosis (Nicoletti et al., 1991). This apoptotic effect of MPA was time-dependent and occurred significantly after 24-h treatment with MPA (Fig. 16B), confirming our previous findings obtained using different methods (Li et al., 1998).

	Fractions			
Treatment time (h)	Sub-G1	G1	S	G2/M
0	4.7 ± 0.4	72.4 ± 1.6	11.0 ± 0.3	13.5 ± 0.5
8	5.5 ± 0.8	74.6 ± 0.5	10.5 ± 0.2	11.2 ± 0.2
16	5.8 ± 0.5	74.5 ± 2.4	9.2 ± 0.6*	9.1 ± 0.4 **
24	11.4 ± 1.5 *	74.3 ± 0.7	6.6 ± 0.3 **	7.7 ± 1.0 **
32	12.3 ±0.2 **	77.8 ± 0.1	4.8 ± 0.1 **	5.6 ±0.2 **
40	34.2 ± 3.0 **	56.7 ± 2.0 *	3.4 ± 0.3 **	5.5 ± 1.1 **
48	44.7 ± 2.8 **	47.6 ± 2.1 **	3.2 ± 0.4 **	5.1 ± 0.6 **

Table 5. Effects of MPA treatment on cell cycle of HIT cells. HIT cells in 6-well plates were treated with 3 μg/ml MPA for indicated time periods. After one gentle rinse, cells were detached by trypsinization and stained with propidium iodide and subject to flow cytometric analysis. Data are expressed as % of total cells (10,000) analyzed. Values are mean ± SE from 3 experiments in triplicate. * P<0.05 and ** P<0.01 *vs*. zero time point.

Cell viability was also assessed by MTS test and DNA content [changes in which were correlated with other typical markers of apoptosis induced by GN-depletion (Li et al., 1998)] every 8 hrs during treatment. MPA led to a dose-dependent decrease of both formazan production (MTS test) and DNA content (data not shown). The time-courses of alterations of these two parameters following MPA treatment of HIT cells were generally parallel, although the decease in DNA occurred slightly before the drop in MTS signal (Fig. 16C), possibly because GTP reduction directly inhibits DNA synthesis (Li et al., 1998; Metz et al., 2001). Significant reductions (-15%, P<0.01) of DNA were observed at 24-hr treatment, a time point when clear apoptotic alterations (chromosome condensation/fragmentation, DNA laddering) occurred (Li et al., 1998). Both formazan production and DNA content were decreased by about 60% after 48-hr exposure to the GN-depleting agent. The time course of changes in MTS test and DNA content after MPA treatment was correlated with the percentage of apoptotic cells (Fig. 16B) as assessed by flow cytometry under the same conditions.

3.2.2. Activation of caspases and release of cytochrome *c* by MPA treatment

The activity of caspase-1 through caspase-10 in HIT cells after MPA treatment was measured by using their respective, specific substrates (Talanian et al., 1997) (Fig. 17). Six such synthetic oligopeptide substrates were used: Ac-WEHD-AFC for caspase-1, -4, and -5; Ac-VDVAD-AFC for caspase-2; Ac-DEVD-AFC for caspase-3, -7 and -10; Ac-VEID-AFC for caspase-6; Ac-LETD-AFC for caspase-8; and Ac-LEHD-AFC for caspase-9. Cell homogenates were prepared after MPA treatment (3 μg/ml) at various time intervals. Activity of several caspases was increased subsequent to sustained GN-depletion. Caspase-2 was clearly activated at 16 hrs, earlier than other caspases and prior to the onset of apoptosis, and maximal activity (+343%) was seen at 32 hrs, prior

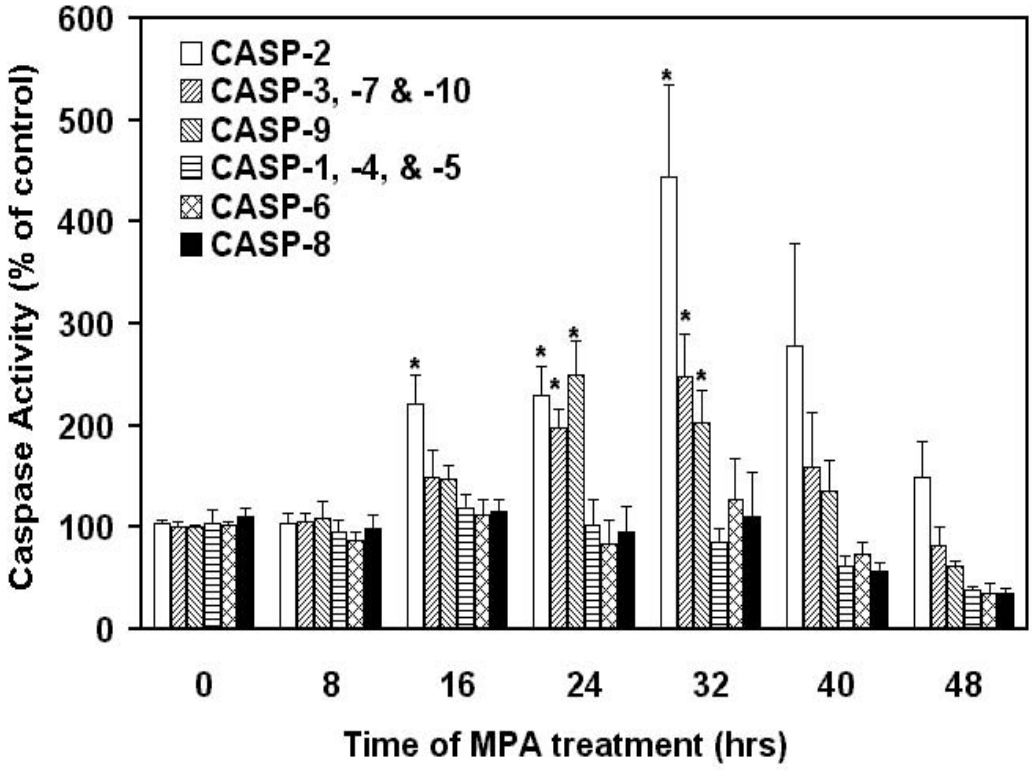

Figure 17. Activity of caspases in HIT cells during MPA treatment. Cells seeded in 6-well plates were treated with 3 μg/ml MPA in culture media for 8-48 hrs. Cell homogenates were prepared as described in detail in the Methods section. Caspase activity was determined by measurement of the cleavage of the fluorogenic, specific substrates. Values are mean ± SE from at least 4 independent experiments. * $P<0.05$ and ** $P<0.01$ *vs.* control

to the maximal levels of apoptosis achieved. In another study measuring caspase-2 activity in shorter intervals (every 2 hrs) during MPA treatment (0-12 hrs), the protease activity was significantly increased by 105% (n=3; P<0.001) at 12 hrs. Caspase-3 (and perhaps also -7 and -10) and caspase-9 were only activated at 24 hrs MPA treatment and their activity was increased maximally by 145% and 150% respectively at 32 hrs (Fig. 17).

Caspase-2 activation was also confirmed by detection of its cleavage (Li et al., 1997b) by Western blotting (Fig. 18). Soane *et al* reported that caspase-2 is activated by cleavage first into three fragments of 32-33 kDa and 14 kDa, thereafter, further processed into 18- and 12-kDa active subunits (Soane et al., 1999). Generally speaking, 32-33 kDa cleavage means caspase-2 activation. Inactivated caspase-2 in control HIT cells displayed a band of 48 kDa on the SDS-polyacrylamide gel. This caspase isoform was dominantly present in cytosol and faintly detected in mitochondria (Fig. 18A). The 48-kDa inactive caspase-2 in cytosol was slightly elevated with the time of culture both in control and MPA-treated cells. After MPA treatment, a band of 35 kDa in cytosol appeared at 16 hrs and reached maximal at 32

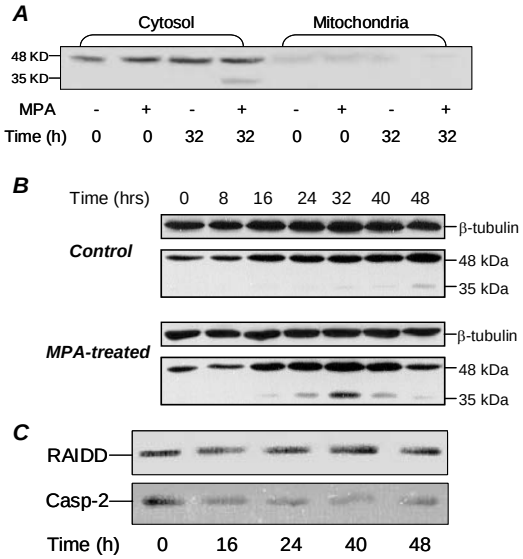

Figure 18. Cleavage of caspase-2 by MPA treatment and interaction of RAIDD with caspase-2. (***A*** and ***B***) HIT cells were treated with 3 μg/ml MPA for 8-48 hrs. Cell lysates were subjected to 10% SDS-PAGE and caspase-2 cleavage was detected by immunoblotting using an antibody reacting with both the enzyme (48 kDa) and its large cleavage product (35 kDa). No difference in the β-tubulin content (loading control) was found between samples. The results were the representative of 5 independent experiments. (***C***) HIT cell homogenates (100 μg protein each) were incubated with anti-RAIDD antibody followed by addition of agarose-immobilized Protein A. After washing and centrifuging, the immunoprecipitation pellets were resuspended in electrophoresis buffer and boiled. The samples were subject to electrophoresis and Western blotting for detection of RAIDD and caspase-2. Data were the representative of 4 independent experiments.

pulled down together with RAIDD (Fig. 18C). Moreover, MPA treatment slightly reduced the amount (a decease of about 20 %; $P<0.05$; n=4) of caspase-2 (inactive form) co-precipitated with RAIDD.

Caspase-9 can be activated by cytochrome *c* (Li et al., 1997b; Green and Reed, 1998), therefore, it is possible that GN-depletion by MPA treatment is capable of causing the release of cytochrome *c* from mitochondria into cytosol. This was shown to be true, since cytochrome *c* in cytosol was increased while that in the mitochondria fraction was decreased in MPA-treated HIT cells (Fig. 19). By analyzing the data from Western blotting with densitometry, the cytochrome *c* content in cytosol was elevated by 57% ($P<0.05$) at 24-hr, and 69% and 174% (both $P<0.01$) after 32 and 48 hrs in corresponding to 18% ($P<0.05$) and 61% ($P<0.01$) reduction of its content in mitochondria. No significant release was observed at 8- and 16-hr treatment. Thus these changes occurred after increase in active caspase-2 but before apoptotic changes (Fig. 16).

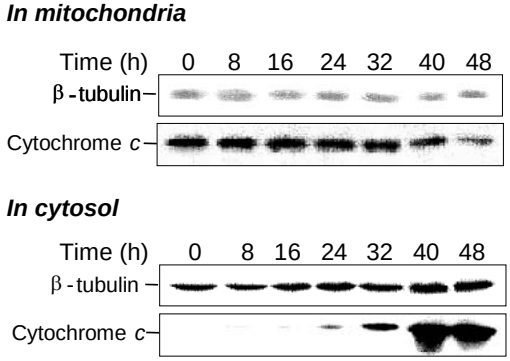

Figure 19. Release of cytochrome *c* from mitochondria into cytosol during MPA treatment. HIT cells were treated with 3 μg/ml MPA for the indicated periods. Mitochondria fraction and cytosol were obtained by subcellular fractionation. They were subjected to 15% SDS-PAGE and cytochrome *c* was determined by immunoblotting. There was no difference in the β-tubulin content between samples. The results were the representative of 4 independent experiments

There was no apparent increase in the activity of caspase-1, -4, -5, -6 and -8 during MPA treatment (Fig. 17).

3.2.3. Blockade of MPA-induced apoptosis by caspase inhibitors

Although GN-depletion activates several caspases, it was unclear whether the latter mediate entirely the apoptosis seen under the same conditions, in view of the reports that apoptosis could occur without involvement of caspases (Borner and Monney, 1999; Lorenzo et al., 1999). Therefore, caspase inhibitors were used to address this question. All cell-permeable caspase inhibitors (10 mM stock solution prepared in DMSO) used below were non-toxic and had no effects on both MTS test and DNA content in HIT cells by themselves under the concentrations applied. A pan-caspase inhibitor, Z-VAD-FMK that suppresses a broad range of the enzymes in the group (MacFarlane et al., 1997) was first used to examine its effects on GN-depletion induced cell death at various concentrations and at different time of treatment (Fig. 20). The decreases in both formazan production (MTS test) and DNA content due to 48-hr MPA treatment were attenuated by 10 μM and were completely restored to normal by 100 μM of the inhibitor (Fig. 20A). When Z-VAD-FMK (100 μM) was added together with MPA to the cells for various periods, the changes both in formazan production (Fig. 20B) and DNA content (Fig. 20C) by GN-depletion was completely blocked at all examined time points.

It is possible that the protective effect of caspase inhibitors on GN-depletion induced apoptosis was mediated via an action on restoration of cellular levels of GTP. The results from 2 series of experiments, however, are against this notion. First, the pan-caspase inhibitor (Z-VAD-FMK, 100 μM) was unable to reverse the inhibition of stimulated insulin secretion in HIT cells treated with MPA for 6 hours (data not shown), a time when levels of GTP are reduced by > 80% (Meredith et al., 1997; Li et al., 2000). By contrast, addition of guanosine was able to restore the GN levels and also reverse the inhibitory effect of MPA on insulin secretion (Meredith et al., 1997;

Li et al., 2000). Second, GN-depletion by MPA activated tissue transglutaminase in HIT cells in a dose- and time-dependent manner (Fig. 8 and Fig. 9). This transglutaminase is an enzyme whose activity is suppressed by mM levels of GTP and may be involved in apoptotic process, in particular the morphological changes. We found that the pan-caspase inhibitor was able to reverse MPA-induced apoptosis, but failed to block the MPA-enhanced tTG activity (Fig. 13). However, co-treatment with guanosine could block both MPA-induced apoptosis and activation of tTG. Therefore, our results clearly indicate that reversal of GN-depletion induced HIT cell death by caspase inhibitors is not trivially due to prevention of the GTP reduction.

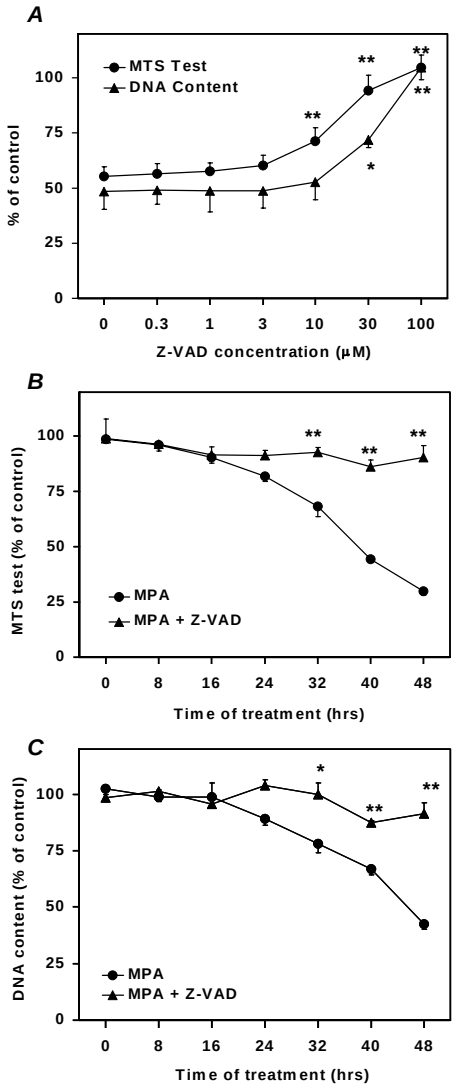

Figure 20. Blockade of GN-depletion induced cell death by a pan-caspase inhibitor. HIT cells in multi-well plates were treated with MPA (3 μg/ml) in the presence or absence of a pan-caspase inhibitor (Z-VAD-FMK) in culture media for various periods. Cell death was assessed by both MTS test and the determination of DNA content in the wells. Values are means ± SE of at least 3 independent experiments. (***A***), Dose-dependent prevention of GN-depletion induced apoptosis by Z-VAD-FMK at 48 hrs of MPA treatment; (***B***) and (***C***), time-course of the protective effects of the pan-caspase inhibitor (100 μM) on cell viability monitored by MTS test and the DNA content. Values are mean ± SE from 4-5 independent experiments. * P<0.05 and ** P<0.01 *vs.* MPA-treatment alone

To define whether a particular caspase(s) plays the critical role in GN-depletion induced apoptosis, specific caspase inhibitors were also used. The caspase-2 inhibitor, Z-VDVAD-FMK (Talanian et al., 1997; Li et al., 1997a; Chen et al., 1999) protected the cells from death due to MPA treatment (Fig. 21). At 100 μM, the caspase-2 inhibitor restored the formazan production almost completely to control values, similar to the action by the pan-caspase inhibitor described above. In addition, the reduction of DNA content could be restored to >80% of control by the caspase-2 inhibitor (this residual reduction, which was still significantly lower than control, may be due to direct inhibition of DNA synthesis by GTP depletion; see above). On the contrary, the caspase-3 inhibitor, DEVD-CHO (100 μM) that blocks apoptosis in other cell types

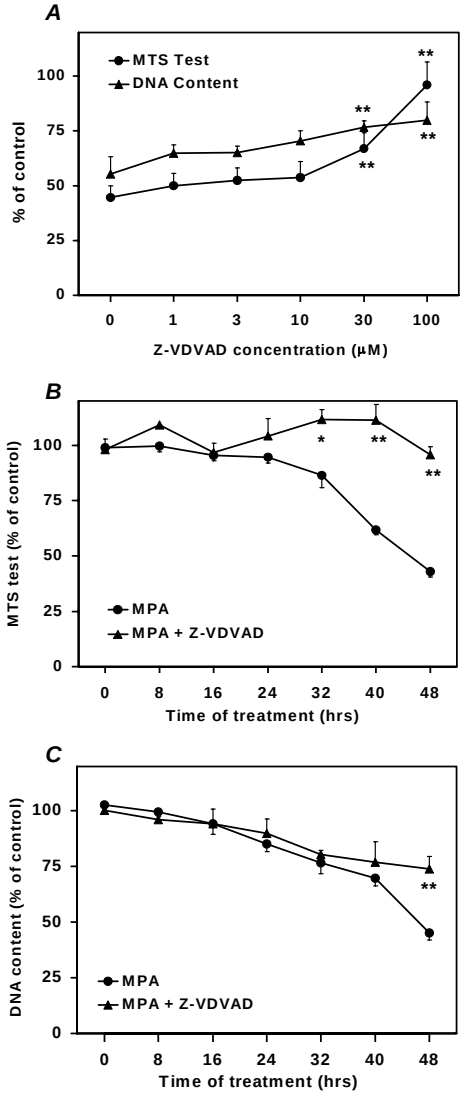

Figure 21. Blockade of GN-depletion induced cell death by a specific caspase-2 inhibitor. HIT cells grown in multi-well plates were treated with MPA (3 μg/ml) in the presence or absence of a specific caspase-2 inhibitor (31) (Z-VDVAD-FMK) for various periods. Apoptosis was monitored by both MTS test and the determination of DNA content. Results are means ± SE of at least 4 independent experiments. (***A***), Dose-dependent blockade of GN-depletion induced apoptosis by Z-VDVAD-FMK at 48 hrs of MPA treatment; (***B***) and (***C***), time-course of the effects of the caspase-2 inhibitor (100 μM) on cell viability assessed by MTS test and the DNA content. Values are mean ± SE from 3-4 independent experiments. * P<0.05 and ** P<0.01 *vs.* MPA-treatment alone.

(Banki et al., 1999; Soane et al., 1999), was only able to partially restore DNA content by 35% and did not restore the cell viability at all during 48-hr MPA treatment (Fig. 22). The caspase-3 inhibitor also failed to reverse the morphological changes due to MPA (data not shown). These findings by using caspase inhibitors suggest that, lthough activated caspase-3 is partially responsible for only the DNA degradation, caspase-2 is a major mediator in the induction of apoptotic death of insulin-secreting cells induced by GN-depletion.

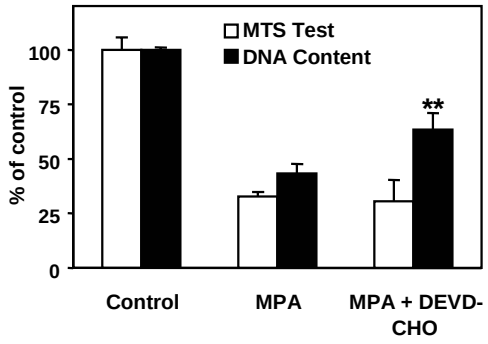

Figure 22. Partial prevention by a caspase-3 inhibitor of reduction of DNA content induced by GN-depletion; comparison to absence of effect on cell viability (MTS test). HIT cells were treated with 3 μg/ml MPA and a caspase-3 inhibitor (DEVD-CHO, 100 μM) for 48 hrs. Results are means ± SE of 3 independent experiments. ** P<0.01 *vs.* MPA-treatment alone.

3.2.4. Section summary

This study investigated the involvement of caspases in GTP-depletion induced apoptosis of β-cells. GTP depletion by MPA treatment reduced cell cycle progress from G1 phase into S and G2/M phases. In addition, activation of several caspases occurred in the process. However, the predominant one with increase in activity is caspase-2 and its activation preceded that of other caspases and, significantly, precedes the appearance of apoptosis. Furthermore, a pan caspase inhibitor and also a specific caspase-2 inhibitor (but not a caspase-3 inhibitor) were able to prevent MPA-induced apoptosis. Release of mitochondrial cytochrome *c* into cytosol and activation of caspase-9 and -3 seems to be down-stream or secondary to caspase-2 activation. These data indicate that activation of caspase-2 mediates the initiation of apoptosis of

pancreatic β cells subsequent to mitogenesis inhibition due to sustained GTP depletion.

3.3. Role of cell cycle regulators in the activation of caspases and induction of apoptosis in β-cells due to GTP-depletion

We have demonstrated above that MPA arrests HIT-T15 cells at G1 phase and triggers caspase-dependent apoptosis in which caspase-2 plays a major role. It appears that a causal relationship between inhibition of mitogenesis and activation of caspases/induction of apoptosis over sustained GTP depletion by MPA may exist. At least two lines of evidence suggest this possibility. First, MPA might affect p53 expression because IMPDH, the specific target of MPA, may interact with p53 (Sherley, 1991; Xu et al., 1998). p53 is an important regulator of cell growth and apoptosis (Vousden et al., 2002). Second, CDK inhibitors, such as $p27^{KIP1}$ and $p21^{WAF1/CIP1}$, play important roles in the regulation of cell cycle progress from G1 phase into S phase (Xiong et al., 1993; Deng et al., 1995; Bissonnette and Hunting, 1998). Our earlier study found that MPA inhibited this transition of cell cycle. It is thus possible that the expression of these proteins may be altered by GN-depletion, resulting in inhibition of mitogenesis and activation of caspases. Consequently, the levels of three cell cycle regulators, p53, $p27^{KIP1}$ and $p21^{WAF1/CIP1}$, were examined in the course of induction of apoptosis by GTP depletion with MPA.

3.3.1. Increment of $p21^{WAF1/CIP1}$ by MPA treatment

GTP depletion by MPA treatment significantly increased $p21^{WAF1/CIP1}$ (Fig. 23A); its mass was elevated by 23%, 74%, 159%, 220% and 94% following 8, 16, 24, 32, and 40 hrs of MPA (3 μg/ml) treatment, respectively. Furthermore, the pattern of time-course of $p21^{WAF1/CIP1}$ induction was closely correlated with that of caspase activation (Fig. 23B) occurring under the same conditions. Both events were significantly apparent at 16 hrs and reached maximal effects at 32 hrs of MPA exposure. These findings suggest that an increase in $p21^{WAF1/CIP1}$ expression may be involved in caspase activation.

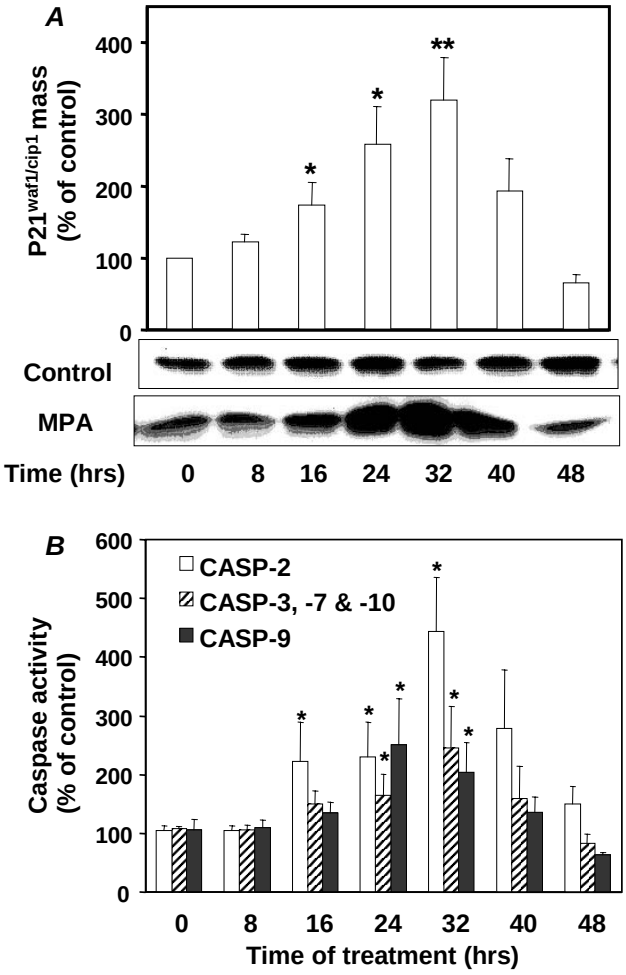

Fig. 23. Close correlation of time-response between the increment of p21$^{Waf1/Cip1}$ mass (*A*) and activation of caspases (*B*) in MPA treated HIT-T15 cells. (*A*), Cells in culture were treated with 3 μg/ml MPA for 0-48 hrs. At the indicated time points, cell lysates were prepared as described in "Materials and Methods". Equal amounts of lysates (20 μg protein extracts) were subject to SDS-PAGE and blotted with an antibody against p21$^{Waf1/Cip1}$. The mass of p21$^{Waf1/Cip1}$ was quantified by computer-assisted densitometry. (*B*), Cells seeded in 6-well plates were treated with 3 μg/ml MPA in culture media for 8-48 hrs. Caspase activity in cell homogenates were determined by measurement of the cleavage of the fluorogenic, specific substrates as described in details in the Methods section. Values are means ± SEM of eight (in *A*) and at least four (in *B*) independent experiments. * $p<0.05$ and ** $p<0.01$ *vs.* zero time-point (in *A*) and *vs.* control (0 μg/ml MPA) (in *B*).

3.3.2. Reduction of p53 and p27^{KIP1} during MPA treatment

Transcription of the p21$^{WAF1/CIP1}$ gene can be activated by both p53-dependent and-independent mechanisms (El Deiry et al., 1993; El Deiry et al., 1994; Agarwal et al., 1995; Gartel et al., 1996; Alpan and Pardee, 1996). Thus we next examined the p53 profile under the conditions when MPA caused an increase of p21$^{WAF1/CIP1}$. Our results revealed that the levels of p53 protein in HIT cells were progressively decreased with the treatment time of MPA (10 μg/ml) (Fig. 24A). After exposure to MPA for 8, 16, 24, 32, 40 and 48 hrs, p53 was reduced by 5%, 11%, 25%, 28%, 34% and 58%, respectively. In addition, this effect occurred in a dose-dependent manner (Fig. 24B).

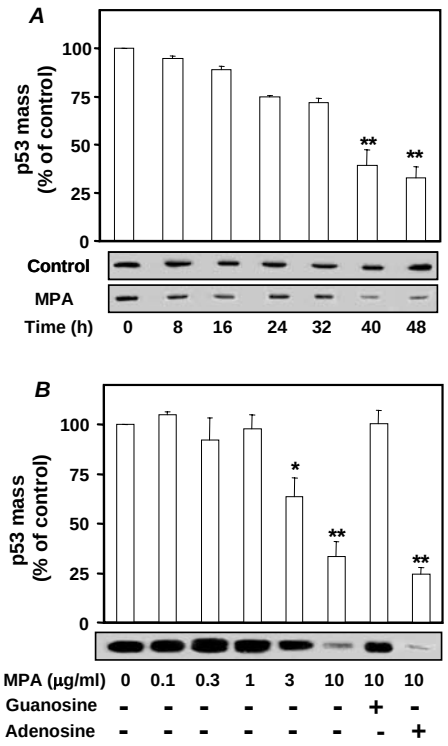

Fig. 24. Reduction of p53 protein by MPA treatment. HIT-T15 cells were cultured in RPMI medium containing 10 μg/ml MPA for 0-48 hrs (*A*) or different concentrations of MPA for 48 hrs (*B*). When present, 0.5 mM guanosine or adenosine was also included. Cell lysates were prepared and subject to SDS-PAGE (20 μg protein each) for separation. Western blotting was performed by blotting the membranes with the sheep polyclonal, peroxidase-conjugated antibody specific for wild p53. The blots were analyzed by computer-assisted densitometry. Values are means ± SEM of five (in *A*) and four (in *B*) independent experiments. * $p<0.05$, ** $p<0.01$ *vs.* zero hour point (in *A*) and *vs.* control (0 μg/ml MPA) (in *B*).

Figure 24. Reduction of p53 protein by MPA treatment

After MPA treatment for 48 hrs, the mass of p53 was significantly decrease by 37% and 64% at 3 and 10 μg/ml, respectively. Co-culture with guanosine (500 μM), but not with adenosine (500 μM), entirely reversed the p53 reduction due to GN depletion by MPA. These observations indicate that the increase of $p21^{WAF1/CIP1}$ by GN-depletion due to MPA treatment in HIT cells is achieved via a mechanism independent of p53.

$p27^{KIP1}$ is another important CKI regulating the progression through G1 and the G1/S transition (Toyoshima and Hunter, 1994; Polyak et al., 1994). MPA treatment caused progressive decreases of the mass of $p27^{KIP1}$ (Fig. 25), in contrast to the increase of $p21^{WAF1/CIP1}$ (cf. Fig. 23). After treatment of 16, 24, 32, 40 and 48 hrs with 3 μg/ml MPA, $p27^{KIP1}$ levels declined by 34%, 55%, 72%, 74% and 75%, respectively (Fig. 25A). This declining effect was also dose-dependent with significant reduction by 41%, 61% and 68% following 48-h treatment at 1, 3 and 10 μg/ml MPA, respectively (Fig. 25B). Thus there is a drop in both p53 and $p27^{KIP1}$ after GN depletion, while the

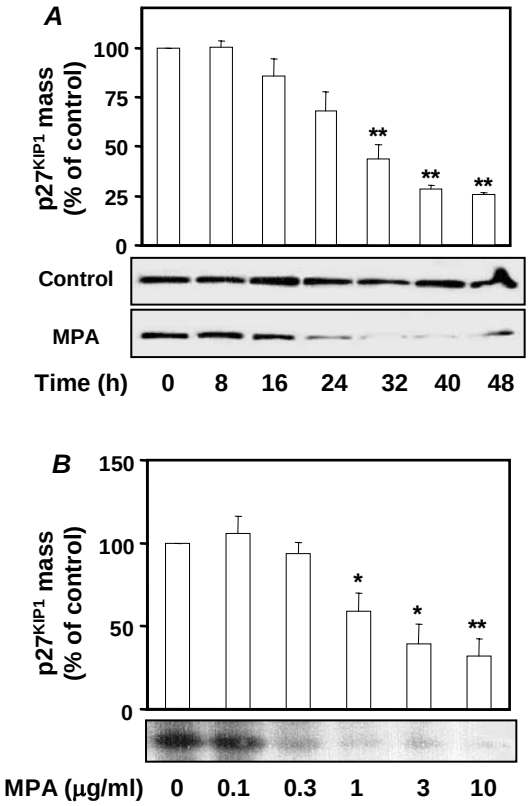

Fig. 25. Decrement of p27^{KIP1} by exposure to MPA in a time- and dose-dependent manner. HIT-T15 cells were treated with 3 μg/ml MPA for 0-48 hrs (*A*) or 0-10 μg/ml MPA for 48 hrs (*B*). Cell lysates (20 μg protein each) were subject to SDS-PAGE and Western blotting was performed by using the goat polyclonal antibody against p27^{KIP1}. The blots were analyzed by computer-assisted densitometry. Values are means ± SEM of three (A and B) independent experiments. * $p<0.05$, ** $p<0.01$ *vs.* zero hour point (in *A*) and *vs.* control (0 μg/ml MPA) (in *B*).

Figure 25. Decrement of p27KIP1 by exposure to MPA in a time- and dose-dependent manner.

latter is a bit more sensitive [occurring at earlier treatment time (32 hrs) and at lower concentrations of MPA (1 μg/ml)].

Interestingly, these MPA effects on p53 and p27^{KIP1} could not be reversed by co-treatment with either a general caspase inhibitor (Z-VAD-FMK) or a specific caspase-2 inhibitor (Z-VDVAD-FMK) (Fig. 26), although both caspase inhibitors are able to

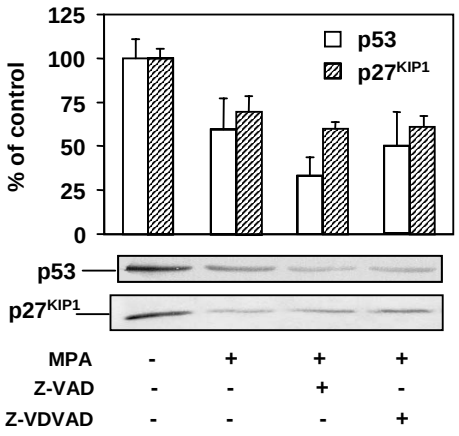

Fig. 26. No restorative effect by caspase inhibitors on MPA-induced reduction of p53 and p27^{KIP1}. HIT cells were treated with 10 μg/ml (for p53) or 3 μg/ml (for p27^{KIP1}) MPA in the presence or absence of a pan-caspase inhibitor (Z-VAD-FMK) or a caspase-2 inhibitor (Z-VDVAD-FMK) in culture for 32 hrs. See legends to Fig. 24 and 25 for other experimental details. Values are means of ± SEM of four (for p53) and ten (for p27^{KIP1}) independent experiments.

block the apoptosis triggered by MPA treatment (cf. Fig. 20 and Fig. 21). These results indicate that GN-depletion by MPA causes decreases of p53 and $p27^{KIP1}$ independent of caspase(s) activation. Since both p53 and $p27^{KIP1}$ were not increased but rather decreased after MPA treatment in a dose- and time-dependent manner, they may not play a major role in the inhibition of mitogenesis due to GN depletion.

3.3.3. Induction of $p21^{WAF1/CIP1}$ and caspase activation

Our above results demonstrated that GTP depletion by MPA induced $p21^{WAF1/CIP1}$ increase in close correlation with caspase activation in a p53-independent mechanism. It is known that $p21^{WAF1/CIP1}$ can serve as a critical checkpoint regulator for both cell cycle arrest and apoptosis (Gervais et al., 1998; Chai et al., 2000). Thus we postulated that $p21^{WAF1/CIP1}$ might mediate the caspase activation occurring during GTP-depletion-induced apoptosis. To test this hypothesis, we utilized a specific inducer of $p21^{WAF1/CIP1}$, mimosine, to mimic the MPA action. This is because mimosine is able to increase both $p21^{WAF1/CIP1}$ mRNA and protein levels independent of p53 (Alpan and Pardee, 1996; Bissonnette and Hunting, 1998), an effect similar to that of MPA. It has been reported that this agent potently causes a reversible block of the cell cycle at late G1 phase and is frequently used to achieve cell synchronization at this stage (Alpan and Pardee, 1996; Ji et al., 1997; Krude, 1999). It was also found that mimosine can interfere with deoxyribonucleotide metabolism and inhibit DNA synthesis (Gilbert et al., 1995). We found that treatment of mimosine for 32 hrs significantly increased $p21^{WAF1/CIP1}$ by 35% and 116% at 100 and 300 μM, respectively (Fig. 27A). Importantly, mimosine (300 μM), mimicking MPA effects, also simultaneously increased activity of caspase-2, -3 and -9 by 220%, 416% and 52%, respectively (Fig. 27B). Furthermore, flow cytometric analysis revealed that mimosine inhibited the progress of cells from G1 to G2/M phase and promoted apoptosis as reflected by DNA

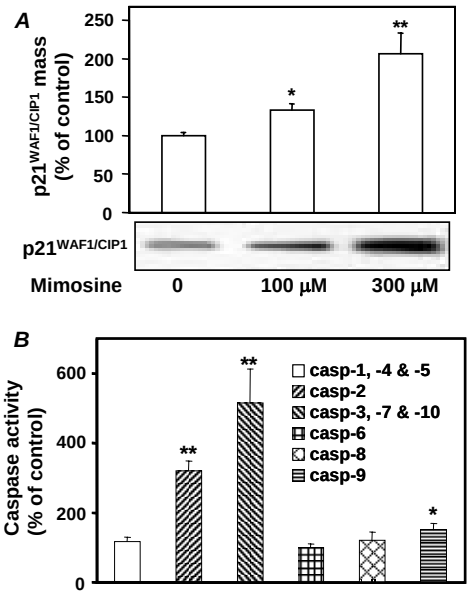

Fig. 27. Induction p21$^{WAF1/CIP1}$ (*A*) and activation of caspases (*B*) by mimosine treatment. HIT cells were treated with mimosine in culture for 32 hrs. The levels of p21$^{WAF1/CIP1}$ in cell lysates (20 μg protein extracts each) were determined by Western blotting and analyzed by computer-assisted densitometry. Caspase activity in cell homogenates were assessed by measuring the cleavage of the fluorogenic, specific substrates as described in details in the "Materials and Methods". Values are means of ± SEM of four (A) and three (B) independent experiments. * $p<0.05$ and ** $p<0.01$ *vs.* control.

fragmentation (an increase in sub-G1 fraction) (Fig. 28A). The number of apoptotic cells (sub-G1) was significantly increased by 1.3 and 5.6 folds ($p<0.01$) after treatment for 32 hrs with 100 and 300 mM mimosine, respectively (Fig. 28B). In addition, cells in S and G2/M phases were significantly reduced by 57% and 74% at 300 mM mimosine (Fig. 28B). Importantly, co-treatment of cells with the pan-caspase inhibitor Z-VAD-FMK (100 μM) could completely block mimosine-induced apoptosis and restore the arrest in cell cycle (Fig. 28A and 6B). However, mimosine-induced cell arrest and apoptosis was not dependent on GTP depletion, since co-treatment of cells with guanosine (500 μM) could not prevent the mimosine effects (data not shown). These data strongly indicate that p21$^{WAF1/CIP1}$ may be the mediator acting as an upstream signal to activate caspases and in turn induce apoptosis during MPA treatment.

It has been reported that p21$^{WAF1/CIP1}$ is a substrate for executive caspases such as caspase-3 and this kind of degradation can be reversed by specific caspase inhibitors

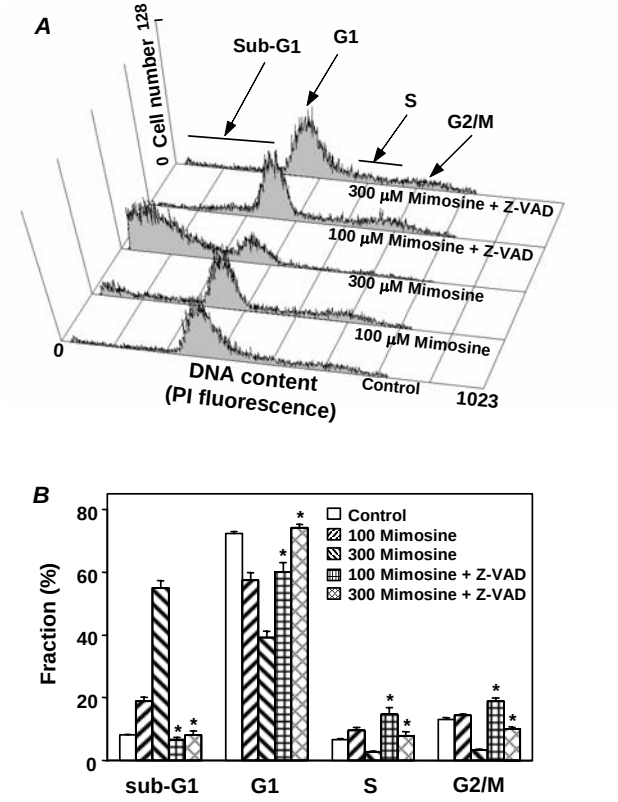

Fig. 28. Blockade by a caspase inhibitor of mimosine-induced apoptosis. HIT cells seeded on 6-well plates were treated with mimosine in culture media for 32 hrs. Cells were stained with PI and subject to flow cytometric analysis of mimosine-induced subdiploid apoptotic cells. The cells whose DNA content was lower than G1 phase (sub-G1) contained fragmented nuclei and were undergoing apoptosis. The graph is a typical representative of 4 independent experiments. The data in (*B)* show the statistical analysis of the mimosine effects on apoptosis and cell cycle. The results are expressed as percentages of total cells (10,000) evaluated by flow cytometry. * P<0.01 *vs.* individual mimosine treatment alone.

(Gervais et al., 1998; Kwon et al., 2002). Similar phenomenon was also observed in our study. We found that the general caspase inhibitor Z-VAD-FMK, but not the caspase-2 inhibitor Z-VDVAD-FMK, enhanced the increment (+38%) of $p21^{WAF1/CIP1}$ due to MPA treatment (Fig. 29). This observation again suggests that $p21^{WAF1/CIP1}$ induction by GTP depletion is an event upstream of caspase activation during apoptosis, although activated caspases may exert a constraining effect on $p21^{WAF1/CIP1}$ levels.

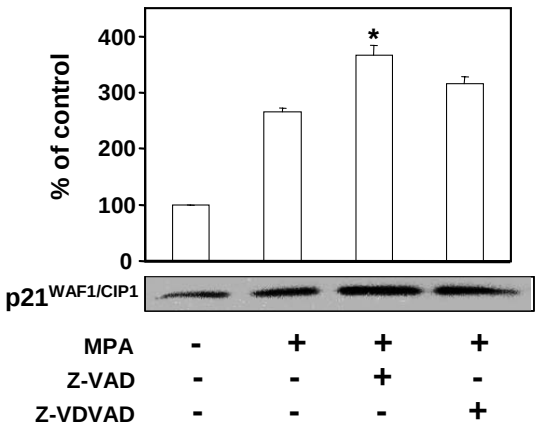

Fig. 29. Enhancement of MPA-induced $p21^{WAF1/CIP1}$ increment by caspase inhibitors. HIT cells were treated with MPA (3 µg/ml) in the presence or absence of a pan-caspase inhibitor (Z-VAD-FMK) or a caspase-2 inhibitor (Z-VDVAD-FMK) in culture for 32 hrs. $p21^{WAF1/CIP1}$ mass in cell lysates (20 µg protein extracts each) was determined and analyzed as described in the legend to Fig. 1. Values are means of ± SEM of thirteen independent experiments. *, p<0.05 *vs.* MPA treatment alone.

3.3.4. Section summary

This portion of work investigated the potential role of three cell cycle regulators (p53, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$) in GTP-depletion induced apoptosis of β-cells, in particular their relationship with caspase activation. We observed that MPA treatment increased protein levels of $p21^{WAF1/CIP1}$, an event which is well correlated with the time course of caspase activation. In contrast, p53 and $p27^{KIP1}$ levels were decreased by MPA treatment. Thus the increase in $p21^{WAF1/CIP1}$ mass and activation of caspases is p53-independent. Moreover, treatment of β cells with mimosine, a p53-independent inducer of $p21^{WAF1/CIP1}$, mimics MPA effects, resulting in $p21^{WAF1/CIP1}$ increase, caspase activation and apoptosis. These data suggest that $p21^{WAF1/CIP1}$ may act as an upstream signal to promote caspase activation and apoptosis in β cells during GTP-depletion.

Chapter 4

Discussion

4.1. The role of tTG in apoptosis induced by GTP depletion

4.1.1. Evidence for association of tTG expression/activation with apoptosis

The relationship between tTG and apoptosis has been broadly investigated in recent years, and it has been proposed that the enzyme may be a complex player of the crucial balance between survival and death (Fesus and Thomazy, 1988; Fesus, 1998; Melino and Piacentini, 1998; Autuori et al., 1998). Several lines of evidence point to a role of tTG in apoptosis. First, a wide range of signaling pathways can lead to the induction of apoptosis associated with increased tTG activity, such as in response to interleukins (Knight et al., 1993), TGF-β (Retter and Davies, 1998), nitric oxide (Bernassola et al., 1999), thapsigargin (increasing intracellular Ca^{2+} levels) (Furuya et al., 1994), and retinoids (Melino et al., 1997; Ritter and Davies, 1998). An augmentation of tTG expression (mRNA and/or protein), accompanied by cross-linking of polypeptides, occurred during apoptosis (Fesus and Thomazy, 1988; Furuya et al., 1994; Melino et al., 1997; Ritter and Davies, 1998). An increase of tTG activity was also observed in isolated human islets undergoing loss of β-cells by apoptosis (Paraskevas et al., 2000). Thus tTG activation is often considered as a marker for apoptosis. Second, substantial knowledge of tTG in apoptosis has been accrued from studies using retinoic acid, a potent tTG inducer (Melino et al., 1997). This agent and its derivatives induced a marked increase (as high as 10-fold) of tTG expression and inhibited cell growth in G1 phase (Piacentini et al., 1993; Oliverio et al., 1999; Ozeki and Tsukamoto, 1999; Ou et al., 2000), often followed by apoptosis (Melino et al., 1997; Oliverio et al., 1999; Ozeki and Tsukamoto, 1999; Ou et al., 2000) which could be blocked by inhibition of tTG activity with tTG inhibitors (Ou et al., 2000). It appeared that retinoids exerted the effects via their nuclear receptors acting on the tTG

promoter (Nagy et al., 1996) and might be mediated by the repression of the expression of bcl-2, c-fos and c-jun (Oliverio et al., 1999; Ozeki and Tsukamoto, 1999), but not of p53 and $p21^{WAF1/CIP1}$ (Ozeki and Tsukamoto, 1999), these proteins are important players in the apoptotic process. Third, experiments overexpressing tTG in fibroblasts and neuroblastoma cells provided further evidence for a role of tTG in the induction of apoptosis (Gentile et al., 1992; Melino et al., 1994). These cells displayed changes in morphology and adhesiveness characteristic of cells undergoing apoptosis as well as enhanced susceptibility to apoptotic stimuli (Gentile et al., 1992; Melino et al., 1994). Consistent with these findings, transfection of cells with tTG antisense markedly decreased both spontaneous and retinoic acid-induced apoptosis (Melino et al., 1994; Autuori et al., 1998; Oliverio et al., 1999). However, it is important to note that most of these studies involved massive over-expression of tTG levels and/or use of stimuli such as retinoids which do not work through changes in GTP, as was the subject of our study.

4.1.2. Increase of tTG activity by GTP depletion *per se* but not by its up-expression in HIT cells

Cell growth is affected by both accumulation (Sidi and Mitchell, 1984) and depletion (Metz and Kowluru, 1999; Cohn et al., 1999; Allison and Eugui, 2000; Metz et al., 2001) of GNs. Under the latter condition, cell growth is inhibited, with drastic reduction in DNA synthesis and alternations in RNA and protein synthesis. We have previously demonstrated that MPA and mizoribine, two structurally-dissimilar IMPDH inhibitors, induce apoptosis of insulin-secreting cells (Li et al., 1998). The current studies investigated the role of tTG in apoptosis using our unique system in which the death of HIT cells is induced by MPA. We hypothesized that since tTG is GTP-dependent (Smethurst and Griffin, 1996; Melino and Piacentini, 1998; Zhang et

al., 1998) and has been implicated in at least the morphological changes of apoptosis (Gentile et al., 1992; Trejo-Skalli et al., 1995; Melino and Piacentini, 1998), it would be a logical candidate to mediate at least a part of the cell death induced by GTP-deficiency. Although 7 transglutaminases have been identified (Aeschlimann et al., 1998), the increase in transamidating activity in MPA-treated β-cells observed in our study appeared due to the activation of GTP-sensitive tTG for two reasons. First, only tTG and epidermal transglutaminase (TG3) are sensitive to GTP; the increased transamidation could be completely prevented by the provision of guanosine (cf. Fig. 9B) which restores the cellular GTP levels. Second, all other transglutaminases except tTG are distributed in specific tissues (Aeschlimann et al., 1998). Our results for the first time revealed the presence of tTG in the β-cells by Western-blotting.

We observed increased *in situ* activity of the enzyme after inhibition of GTP synthesis. In contrast to the observations of increased tTG expression in other studies (Furuya et al., 1994; Melino et al., 1997; Ritter and Davies, 1998), however, this increment of tTG activity by MPA was not associated with an augmented expression of tTG protein (rather even with reduced mass at late treatment time periods) in our study. This phenomenon indicated that MPA altered tTG activity by lowering intracellular GTP level and not by increasing its synthesis, since tTG activity is negatively regulated by intracellular GTP levels (Smethurst and Griffin, 1996; Melino and Piacentini, 1998; Zhang et al., 1998). This notion was further supported by our experiments using guanosine which, by restoring GNs to normal levels (Metz et al., 1992; Meredith et al., 1997; Li et al., 2000), blocked the increase of tTG activity and prevented HIT cells from apoptosis due to MPA. An increase of tTG activity during apoptosis but unaccompanied by amplified expression of tTG has also been observed in other studies (Korner et al., 1993; Holmes and Haynes, 1996).

tTG acts not only intracellularly but also can be released by externalization to extracellular space there tTG facilitates the assembly and remodeling of extracellular matrices and promotes cell adhesion (Gaudry et al., 1999a; Gaudry et al., 1999b; Verderio et al., 1999; Balklava et al., 2002; Rosenthal et al., 2003; Gross et al., 2003). Though not directly examined, such phenomena might occur also in our MPA-treated HIT cells, which could account for the reduction of tTG mass at late treatment time periods.

4.1.3. tTG activation is an epiphenomenon during MPA-induced apoptosis of HIT cells

Importantly, we observed that the time-course of the increase of *in situ* tTG activity was paralleled with the induction of programmed cell death. Consequently, whether tTG is an upstream effector in the induction of GN-depletion induced apoptosis was examined by using its inhibitors. It has been reported that tTG inhibitors block apoptosis induced both by retinoic acid (Ou et al., 2000) and by expressing truncated DRPLA (dentatorubral-pallidoluysian atrophy) protein (Igarashi et al., 1998). In our study, two specific tTG inhibitors (MDC or putrescine) (Tsai et al., 1998) reduced MPA-evoked tTG activity by ~43%. However, the inhibitors were barely able to relieve cell death caused by MPA treatment. In addition, tTG activity is also regulated by intracellular Ca^{2+} levels (Smethurst and Griffin, 1996; Melino and Piacentini, 1998; Zhang et al., 1998). Although the enzyme is not active at Ca^{2+} levels normally detected in viable cells (<1 μM) (Folk, 1980), the apoptosis-associated rise in cellular Ca^{2+} concentrations is sufficient to activate it (Fau et al., 1997). We have previously observed that even 6-hr MPA treatment raised resting intracellular Ca^{2+} levels by 20% in HIT cells (Li et al., 2000). It is thus very likely that higher Ca^{2+} levels would be reached in these cells after longer MPA treatment, which may contribute to tTG

activation. Therefore, a Ca^{2+} chelator was used to lower extracellular Ca^{2+} to 80-120 nM (much lower than 1 μM that activates tTG). This maneuver could almost abolish the increased tTG activity due to GN depletion by MPA treatment, but was still only capable of partially protecting the cells from death. Thus these experiments inhibiting tTG did not clearly reveal a biologically-significant role for this enzyme as an "initiator" of cell death induced by GTP-depletion. In a study by Zhang *et al*., MDC prevented the increase in the cross-linked envelopes caused by tTG activation, but failed to inhibit DNA fragmentation due to interferon-β (Zhang et al., 1999a). Therefore, it seems that the major role of tTG in apoptosis is to function as a downstream effector in the late phase of apoptosis (Fesus, 1998; Melino and Piacentini, 1998). By catalyzing the Ca^{2+}-dependent formation of an insoluble protein scaffold from stable cross-links between proteins during apoptosis (Fesus and Thomazy, 1988; Fesus, 1998; Melino and Piacentini, 1998; Autuori et al., 1998), tTG could maintain the contents of the dying cells before their clearance by phagocytosis, and thus prevent the non-specific leakage of harmful intracellular components (e.g. lysosomal enzymes, nucleic acids, etc.) and consequently inflammatory responses (Melino and Piacentini, 1998). We found that 48-h exposure to MPA caused a marked increase of LDH release. However, neither a combination of two tTG inhibitors (MDC and putrescine) nor lowing Ca^{2+} by EGTA affected the MPA-induced LDH release. These findings suggest that tTG is also unlikely to be involved in the terminal release of large molecular compounds from the apoptotic cell. Our findings indicate that although an increase of tTG activity may accompany apoptosis, the activation of this enzyme may be not essentially required for the induction or execution of at least a significant number of the changes seen during apoptosis.

4.1.4. Role of tTG in GTP depletion-induced apoptosis of HIT cells

The possible role of tTG in maintaining cell integrity for apoptotic cells to avoid the release of cellular components leading to inflammation was proposed but is not well defined. The liver cells undergoing apoptosis induced extensive cross-linking of cellular proteins resulting in the formation of SDS-insoluble shells (similar to cornified envelopes of epidermis) in the so-called "apoptotic bodies" that contain oligonucleosomal fragments (Fesus et al., 1989). The insolubility of these apoptotic bodies was evoked by protein cross-linking bonds formed by tTG (Fesus et al., 1989). A later study observed that an increase of tTG activity in hepatocytes was paralleled by formation of SDS-insoluble apoptotic bodies (Piacentini et al., 1991). In addition, intense tTG immunostaining is also found in the apoptotic bodies present inside phagosomes within the cytoplasm of neighboring cells (Piacentini et al., 1991). Thus cross-linking proteins by tTG plays a role in the formation of apoptotic bodies ready to be cleaned by the engulfing cells. Many substrates of tTG are cytoskeletal proteins and some are nuclear proteins such as Rb (Melino and Piacentini, 1998), but there is no evidence that LDH is a tTG substrate. Published studies did not reveal a relationship between LDH release and tTG activity in apoptotic cells in which the tTG was either knocked out or highly expressed (De, V and Melino, 2001; Tucholski and Johnson, 2002). In one study, however, the number of cross-linked apoptotic bodies was significantly reduced in the tTG knockout cells (De,Laurenzi and Melino, 2001). The postulated role of tTG in the maintenance of cell integrity may occur in a critical period during the apoptotic process, i.e. DNA fragmentation and formation of apoptotic bodies before being engulfed by neighboring cells. In MPA-treated HIT cells, DNA fragmentation occurred after 24-h treatment (Li et al., 1998). Taken together, the data suggest that while tTG is unlikely to mediate cytoplasmic leakage in

terminally apoptotic or necrotic cells (at least of large molecular weight molecules such as LDH), cross-linking of proteins by tTG may well play an important role in the morphological changes during programmed cell death. For the latter effect, cytoskeletal proteins (actin, microtubules and intermediate filaments) may be implicated since they act not only as the anchorage sites but also the substrates for tTG in cells undergoing apoptosis (Trejo-Skalli et al., 1995; Melino and Piacentini, 1998; Piredda et al., 1999). We indeed observed that the alterations of HIT cell morphology which accompanied tTG activation during MPA treatment were blocked by guanosine and partially relieved by tTG inhibitors. We emphasize however, that we cannot exclude the possibilities that other roles for tTG might have been unmasked if we had been able to inhibit it completely, or if we had studied different inducers of apoptotic cell death.

4.1.5. Relationship between tTG and caspases

It is now known that caspases play an essential role in both initiation and execution of apoptosis (Green and Reed, 1998; Budihardjo et al., 1999). Many of tTG-targeted proteins are also substrates of caspases (Autuori et al., 1998) and thus there may exist a relationship between tTG and caspases. In our study, tTG activation by GTP-depletion was not affected when a pan-caspase inhibitor blocked activity of all caspases (Huo et al., 2002). More importantly, our data indicated that the rise in tTG activity was not merely secondary to the apoptosis induced by GTP depletion, since the pan-caspase inhibitor prevented the cell from death but did not suppress the augmented tTG activity. Alternatively, this observation cannot completely rule out the possibility that tTG activation is an upstream event of caspase activation in GTP-depleted cells; the unavailability of more potent tTG inhibitors impedes the assessment of this possibility. However, the morphological changes evoked by GTP-depletion

could not be completely reversed by the caspase inhibitor, allowing for the possibility of an additional important role of tTG in this aspect. This concept was further substantiated by the observation that tTG inhibitors were able to partially prevent MPA-induced changes in cell morphology, corresponding to the partial suppression of the enhanced tTG activity under such conditions. Interestingly, tTG is a caspase-3 substrate and is cleaved during apoptosis in lymphoid cells (Fabbi et al., 1999). Cleavage of tTG causes loss of its cross-linking function, and this event was regarded as a valuable biochemical marker of caspase 3 activation during the late execution phase of apoptosis. This may explain our observation of reduction of tTG mass in HIT cells at late MPA treatment (48 hrs) following activation of caspases. Alternatively, the reduction of tTG mass might be due to the activation of other proteases, since it has been reported that, in neuroblastoma SH-SY5Y cells, depletion of GTP increases tTG degradation by calpain after elevation of intracellular Ca^{2+} levels (Zhang et al., 1998), a phenomenon perhaps also occurring in our HIT cells (Li et al., 2000).

In conclusion, tTG may play a role as a downstream regulator in GN-depletion induced apoptosis in HIT cells, but this role seems to be principally involved to the terminal morphological changes. Interestingly, it has been reported that an increase of tTG activity was not observed during apoptosis under some circumstances, such as by treatment with dexamethasone (Johnson et al., 1998), peroxinitrate (Virag and Szabo, 2000), and by Fas-receptor stimulation (Szondy et al., 1997). Moreover, apoptosis induced by various agents were not affected in tTG "knock-out" cells (De, V and Melino, 2001). These studies, in conjunction with the current ones, suggest that the hypothesis that tTG is a universally crucial component of the main pathway of the early apoptotic programs remains to be substantiated.

4.2. Caspase activation in β-cell apoptosis induced by GTP depletion

4.2.1. Activation of multiple caspases in MPA-induced apoptosis of HIT cells

Our data strongly suggest that HIT cell apoptosis due to GN-depletion was mediated by activation of at least two initiator caspases (caspase-2 and caspase-9) and at least one effector caspase (caspase-3). In addition, caspase-3 activation occurred later than caspase-2 and a specific caspase-2 inhibitor also blocked caspase-3 activation, indicating that the latter is activated subsequent to the former. Caspase-3 is a downstream effector directly killing cells in some circumstances (Banki et al., 1999; Soane et al., 1999), but there is increasing evidence that caspase-3 inhibition fails to block apoptosis in other systems (Tepper et al., 1997; Bras et al., 1999). In our study, a caspase-3 inhibitor failed to prevent the β-cell from death induced by GTP-depletion; however, it exerted some protective effect on DNA reduction which may suggest some role in the latter process. This also suggests that other effector caspases play important roles, along with caspase-3, in the final execution of cell death.

4.2.2. Caspase-2 activation is prior to cytochrome *c* release and caspase-9 activation

We found that caspase-2 in HIT cells was mainly localized in cytosol and only a small portion was detected in mitochondria-rich fraction. It has been proposed that caspase-2 and -9 zymogens and cytochrome *c* essentially localized in mitochondria may be released and become activated, eventually triggering of apoptosis (Green and Reed, 1998; Li et al., 1997b; Ito et al., 1999; Banki et al., 1999; Susin et al., 1999a). Thus the integrity of mitochondria may be critical for subcellular redistribution and activation of these caspases. In this study, we could not find detectable changes of caspase-2 content in mitochondria after GN-depletion, suggesting that the activated caspase-2 is not mainly derived from mitochondria. Although we did observe the leakage of

cytochrome *c* from mitochondria and modest activation of caspase-9 (indicating a damage of the organelles) occurred in GN-depleted β-cells, these events took place later than the increase of caspase-2 activity. In addition, a caspase-2 inhibitor was able to suppress the activation of both caspase-2 and caspase-9. All these results suggest that cytochrome *c* release and activation of caspase-9 are delayed or secondary phenomenon and the damage to mitochondria might not be the essential event in the triggering of apoptosis induced by sustained GN depletion.

4.2.3. Activation of caspase-2 mediates β-cell apoptosis due to GN-depletion

Caspase-2 is involved in the apoptosis under a variety of conditions. It is activated during cell death induced by TNFR in B-lymphocytes and other cells (Thome et al., 1998; Shearwin-Whyatt et al., 2000), but the major mediator of TNFR is caspase-8 as the deletion of RAIDD (caspase-2 adaptor) failed to block TNF-induced apoptosis (Duan and Dixit, 1997). Caspase-2 activation is also required for the apoptosis induced by withdrawal of trophic support from PC cells and sympathetic neurons (Stefanis et al., 1999) and caspase-3-like activity is not required in this paradigm. TGFβ-evoked apoptosis of a variety of cells also involves caspase-2 activation (Choi et al., 1998). Thus it is highly possible that the antecedent blockade of mitogenesis (Fig. 16A and Table 5) subsequently induces apoptosis (Fig. 16B, C) to prevent accumulation of DNA-aberrant cells in G1/S phases. It is interesting to note that caspase-2, similar to its effect in GN-depletion-elicited HIT cell death, plays a major role in apoptosis in B-lymphocytes induced by B cell receptor (BCR) cross-linking (Chen et al., 1999). In the latter system, caspase-2 was activated early and markedly while caspase-9 activity was moderately enhanced without the involvement of caspase-8. In addition, the cell death could be prevented by pan-caspase inhibitors or specific caspase-2 inhibitor, resembling the findings in GN-depletion induced apoptosis of HIT cells. Thus

caspase-2 activation may represent another major class of apoptotic pathways distinct from that mediated by caspase-8 and caspase-9 (Kumar, 1999; Earnshaw et al., 1999).

4.2.4. Possible mechanism for caspase-2 activation in β-cell by GN-depletion

How does GN depletion activate caspase-2 in β-cells? Although it might involve the inhibition of primer RNA biosynthesis (Catapano et al., 1995), reductions in mitogenic competence, and/or the interference with the function of some small G-proteins (Kowluru et al., 1996; Li et al., 1998), the exact nature of the upstream mediators leading to the observed activation of caspase-2 is unclear. It has been proposed that RAIDD may be an upstream regulator of caspase-2 since an interaction between the two proteins occurs both in the cell and in cell-free system (Duan and Dixit, 1997; Shearwin-Whyatt et al., 2000). Endogenous RAIDD is mainly localized in the cytoplasm and to some extent in the nucleus, where its interaction with caspase-2 may occur (Shearwin-Whyatt et al., 2000). We also found that RAIDD interacts with caspase-2 in HIT cells, as assessed by immuno-precipitation. It appeared this interaction was slightly reduced by GN-depletion. What importance of this change to GN-depletion induced apoptosis is unclear in the moment since there is no study which reported an alteration of RAIDD-caspase-2 interaction during apoptosis.

Our other preliminary studies may shed some light on the mechanism underlying caspase-2 activation by GN-depletion. It is known that the molecules involved in the regulation of cyclin-dependent kinases (CDKs) (Matsumoto et al., 1999), such as p53 and $p21^{WAF1/CIP1}$, play an important role in the control of cell survival and death (Jayaram et al., 1999; Zhang et al., 1999b). p53 is a tumor suppressor that regulates $p21^{WAF1/CIP1}$ and its expression is often increased in apoptotic cells (Wu et al., 1998; Zhang et al., 1999b). Cdk4 is particularly important for islet β-cell growth and post-natal survival (Rane et al., 1999). Induction of $p21^{WAF1/CIP1}$ is observed in the islets

under growth arrest and death due to exposure to oxygen radicals (Kaneto et al., 1999a). Similar increases in $p21^{WAF1/CIP1}$ and also p53 occur in amylin-induced β-cell death (Zhang et al., 1999b). It is of particular interest that p53 may regulate IMPDH (and vice versa) (Sherley, 1991), the key enzyme in GTP biosynthesis which is targeted by MPA. Nonetheless, p53 seems unlikely to play a major role in GN-depletion induced apoptosis as its mass was reduced after MPA treatment (Fig. 24). Interestingly, however, our results (Fig. 23) indicated that $p21^{WAF1/CIP1}$ was up-regulated in close parallel with caspase-2 activation, suggesting that $p21^{WAF1/CIP1}$ might be involved in the initiation of cell death due to GN depletion. Moreover, the increase in $p21^{WAF1/CIP1}$ during GN-depletion could not be blocked by caspase inhibitors which prevented β-cells from death, suggesting that $p21^{WAF1/CIP1}$ might be an upstream signal for caspase-2 activation (see more discussions later in the Section 4.3).

4.2.5. Involvement of caspases in β-cell death caused by harsh challenges

Both clinical and experimental studies indicate that a failure of islet β-cell function, arrest of cell growth and promotion of cell death by apoptosis occur during challenge with cytokines, glucose toxicity or lipo-toxicity (Shimabukuro et al., 1998; Pick et al., 1998; Kay et al., 2000). Cytokines such as IL-1β, TNF and interferon-γ induce damage and induce β-cell death via Fas and TNFR (Iwahashi et al., 1996; Delaney et al., 1997; Stephens et al., 1999). Similar apoptotic effects on β-cells are also seen subsequent to the stimulation of amyloid (Zhang et al., 1999b) or free radicals such as nitric oxide generated from streptozotocin (Kaneto et al., 1999b). However, the information on caspases in the death of islet β-cells occurring in the above circumstances is very fragmentary. Our unpublished data indicate that caspase-2 may be not the critical protease in all forms of programmed cell death, since the caspase-2 inhibitor (Z-

VDVAD-FMK) could not prevent the apoptotic death of HIT cells induced by streptozotocin and only partially (by ~30%) reversed cytokine (IL-1β plus TNFα)-induced HIT cell death. It has been reported that IL-1β lowered GTP by ~60% in islet β-dells (Meredith et al., 1996). Other studies reported the activation of caspase-1 and -3 by cytokines in β-cells (Stephens et al., 1999; Karlsen et al., 2000). These observations suggest again that caspase-2 activation may play a specific, critical role in the apoptosis induced by GN-depletion with MPA, due to impairment of mitogenesis. Little is known about the role of caspases in β-cell apoptosis caused by glucose toxicity and lipid-toxicity. Our preliminary results indicated that high concentrations of free fatty acid increased the activity of both caspase-2 and caspase-3 in insulin-secreting INS-1 cells (data not shown). The present study on β-cell growth and death during GTP-depletion may enrich our knowledge in this aspect.

In conclusion, this is the first study reporting that multiple caspases (including at least caspase-2, -3, and -9) were activated and the release of mitochondrial cytochrome *c* occurred during apoptosis of β-cells induced by GTP depletion with MPA. Importantly, caspase-2 like protease(s) was activated earlier and more dramatically than other caspases. Furthermore, GTP-depletion induced apoptosis and caspase activation were blocked by a pan-caspase inhibitor and, significantly, protected by a specific caspase-2 inhibitor as well, pointing a major role of the latter in the initiation of apoptotic death of insulin-secreting HIT cells. Thus our study revealed a novel, caspase-2 mediated form of apoptosis that may be consequent to impaired mitogenesis as demonstrated by flow cytometry in the current study and by thymidine/BrdU incorporation previously (Li et al., 1998; Metz et al., 2001).

4.3. Role of cell cycle regulators in β-cell apoptosis induced by GTP depletion

We have observed that specific GTP depletion by MPA restrained mitogenesis of insulin-secreting cells by reducing their progression from G1 phase into S and G2/M phases, resulting in apoptosis mediated by activation of caspases (Li et al., 1998; Huo et al., 2002). We further defined the linkage between cell cycle arrest and the induction of apoptosis induced by MPA treatment in HIT cells. Our data indicate that MPA induced $p21^{WAF1/CIP1}$ expression which was closely correlated with activation of caspases. Furthermore, a specific $p21^{WAF1/CIP1}$ inducer, mimosine, could mimic the MPA effects on activation of caspases resulting in apoptosis (but in GTP-independent manner). To our knowledge, this is the first study systematically investigating $p21^{WAF1/CIP1}$ and establishing its relationship with caspase activation during apoptosis induced by GTP depletion.

4.3.1. Close relationship between $p21^{WAF1/CIP1}$ and caspase activation in β-cells

It is well known that CKIs are able to inhibit cell proliferation by negatively affecting cyclin-CDK complexes. By doing so, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ regulate the progression through G1 and the G1/S transition (Toyoshima and Hunter, 1994; Polyak et al., 1994). Since depletion of cellular GNs by MPA inhibits cell proliferation and arrests cell cycle in G1 phase (Mitchell et al., 1993; Laliberte et al., 1998; Huo et al., 2002), it is possible that MPA may alter $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ expression to achieve this effect. Only one study has mentioned the induction of $p21^{WAF1/CIP1}$ by MPA (1 μM) in T lymphocytes and this effect occurred at 42-h, but not 24-h, treatment with MPA (Laliberte et al., 1998). In the present study, we found that depletion of GNs by MPA treatment caused increases of $p21^{WAF1/CIP1}$ following a time-course which was in parallel with the activation of several caspases (cf. Fig. 23), suggesting a close

relationship between the two events. Importantly, significant accumulation of $p21^{WAF1/CIP1}$ and activation of caspases were observed as early as 16 hrs of MPA treatment and thus preceded the occurrence of apparent apoptosis (at 24 hrs) of HIT cells (Li et al., 1998; Huo et al., 2002).

To further examine the possible sequence of observed caspase activation and $p21^{WAF1/CIP1}$ accumulation, we used mimosine, a well-known $p21^{WAF1/CIP1}$ inducer, to study its effects on the two events. By increasing $p21^{WAF1/CIP1}$, this compound has been found to be a potent, reversible blocker synchronizing cells in late G1 phase (Alpan and Pardee, 1996; Ji et al., 1997; Krude, 1999). Mimosine increased both $p21^{WAF1/CIP1}$ mRNA and protein levels bypassing the requirement for transcriptional activation by p53 (Alpan and Pardee, 1996; Bissonnette and Hunting, 1998). However, there is no study reporting its effect on caspases and apoptosis directly. In the current study, we found that mimosine had effects similar to those of MPA in increasing $p21^{WAF1/CIP1}$ and inducing activation of same types of caspases (Fig. 27). Moreover, under these conditions, mimosine suppressed cell cycle and promoted caspase-mediated apoptotic death of HIT cells, the same effects observed after MPA treatment (Fig. 16). Although it has been reported that mimosine inhibits deoxyribonucleotide metabolism (Gilbert et al., 1995), we found that its effects on cell arrest and apoptosis are not due to a depletion of GTP. Our experiments using caspase inhibition further uncovered the relationship between $p21^{WAF1/CIP1}$ and caspase activation. When caspases were suppressed by a pan-caspase inhibitor, $p21^{WAF1/CIP1}$ mass was even modestly enhanced during MPA treatment (Fig. 29), indicating that GN depletion can induce $p21^{WAF1/CIP1}$ without caspase activation, in other word, prior to caspase activation. These observations strongly suggest $p21^{WAF1/CIP1}$ may act as an upstream signal to activate caspases. Therefore, we can infer that sustained GTP-depletion significantly increased

$p21^{WAF1/CIP1}$ accumulation which triggered a cascade of activation of caspases leading to apoptosis since GTP repletion reversed these effects.

4.3.2 Activation of caspase(s) and $p21^{WAF1/CIP1}$ degradation

The observation of enhanced $p21^{WAF1/CIP1}$ accumulation in the presence of a pan-caspase inhibitor also implies that $p21^{WAF1/CIP1}$ may be degraded by activated caspases. Indeed, there is evidence that $p21^{WAF1/CIP1}$ may be a substrate of effector caspases in the late stage of apoptosis (Chai et al., 2000; Kwon et al., 2002). The significance of this phenomenon, however, is unclear. It might be a negative feedback mechanism whereby the cell protects itself from damage by shutting off the upstream signals.

4.3.3. Induction of p53-independent $p21^{WAF1/CIP1}$ increment by MPA

Ample evidence demonstrated that induction of $p21^{WAF1/CIP1}$ can be achieved by either p53-dependent (El Deiry et al., 1993; El Deiry et al., 1994) or -independent pathways (Agarwal et al., 1995; Alpan and Pardee, 1996). In lymphocytes 24-h treatment with MPA (1 μM) resulted in a very low level of p53 expression (Laliberte et al., 1998). In our study, the $p21^{WAF1/CIP1}$ induction was apparently not mediated by p53, since the latter was progressively reduced by MPA treatment in both time- and dose-dependent manner. Thus p53 may not be an important mediator for MPA-induced apoptosis.

IMPDH is a key enzyme for biosynthesis of GNs and its gene expression is regulated inversely by a posttranscriptional nuclear event in response to fluctuations in the intracellular level of GNs (Glesne et al., 1991). As a specific inhibitor of IMPDH, MPA diminished GNs levels and, therefore increased levels of IMPDH mRNA and amounts of the enzyme (Glesne et al., 1991; Metz et al., 2001). In addition, IMPDH activity and its protein and mRNA levels could be down-regulated by p53 and thus this enzyme (or the resultant changes in cellular levels of GNs) might play an important role in mediating p53-dependent suppression of cell growth (Sherley, 1991;

Liu et al., 1998b; Jayaram et al., 1999; Yalowitz and Jayaram, 2000). The reciprocal effect of IMPDH on p53 has not been reported. However, our findings that p53 was decreased by GTP depletion [which would increase IMPDH expression (Metz et al., 2001)] suggested such a possible consequence. This notion was further supported by the results obtained using caspase inhibitors. Under these conditions, caspase activation and apoptosis due to MPA were blocked while GNs remained depleted (Huo et al., 2002) and the effect of reducing p53 was not affected (cf. Fig. 26A). Only restoration of GNs by guanosine was able to prevent p53 reduction (cf. Fig. 24B). Thus we can conclude that p53 decrement in HIT cell during MPA-induced apoptosis is attributable to GTP depletion. Alternatively, the reduction of p53 mass may be due to the degradation by some unknown proteases activated by GN depletion.

4.3.4. $p27^{KIP1}$ degradation by MPA treatment

Little information on the effect of GN on $p27^{KIP1}$ is available. One study reported that MPA prevented the IL-2-induced elimination of $p27^{KIP1}$ and resulted in the retention of high levels of $p27^{KIP1}$ in IL-2/leucoagglutinin-treated T cells (Laliberte et al., 1998). Our data revealed that MPA-induced arrest of HIT cells may not be mediated by $p27^{KIP1}$ since the mass of this CKI was actually reduced by MPA in a dose- and time-dependent manner. In addition, $p27^{KIP1}$ was not a substrate of caspases activated by GN depletion, since the reduction of $p27^{KIP1}$ was not reversed by caspase inhibitors, suggesting an effect possibly related to GN depletion directly. Whether this reduction was due to decreased expression or to increased degradation of the protein is unclear. The level of $p27^{KIP1}$ is regulated in several ways (Slingerland and Pagano, 2000), the precise biochemical link between GTP depletion and $p27^{KIP1}$ reduction in islet β-cells remains to be clarified in further study. Interestingly, in this study we found that both p53 and $p27^{KIP1}$ were decreased (though the latter was more sensitive) following MPA

treatment; but it is not known whether any relationship exists between the two events based on the data from this study and the studies reported in the literature.

4.3.5. Cell cycle and apoptosis in β-cells

Cell cycle molecules play important roles in islet β-cell growth and death and the development of diabetes. It has been found that Cdk4 (which forms a complex with cyclin D1 and is required for the progression of cell cycle from G1 to S phase) is essential for islet β-cell growth and postnatal survival and that the loss of its expression causes insulin-deficient diabetes in laboratory models (Rane et al., 1999; Tsutsui et al., 1999). Degeneration of pancreatic islets by apoptosis also occurs in the Cdk4 knockout mice after birth (Tsutsui et al., 1999). In addition, there is evidence that induction of $p21^{WAF1/CIP1}$ expression is involved in islet β-cell death caused by oxidative stress (Kaneto et al., 1999a), which may contribute to glucose toxicity. In another study, enhanced expression of p53 and $p21^{WAF1/CIP1}$ occurred in amylin-induced apoptosis of insulin-secreting cells (Zhang et al., 1999b). Our findings in this study and the results by others (Kaneto et al., 1999a; Zhang et al., 1999b) have suggested that $p21^{WAF1/CIP1}$ may play an important role of in the regulation of islet β-cell apoptosis (Fig. 30).

In conclusion, our results demonstrated that depletion of GNs in HIT cells induced p53-independent up-expression of $p21^{WAF1/CIP1}$, an effect closely correlated with the time-couse of activation of caspases and induction of apoptosis. In addition, an increase of $p21^{WAF1/CIP1}$ was still observed when caspase activation was suppressed. Both p53 and $p27^{Kip1}$ seem not to play an important role in the apoptosis induced by

MPA treatment, since they were degraded during the process. A selective inducer of $p21^{WAF1/CIP1}$ mimicked the MPA effects, causing caspase activation and apoptosis

which could be blocked by a pan-caspase inhibitor. These observations suggest that an induction of $p21^{WAF1/CIP1}$ may mediate the arrest of cell cycle, activation of caspases and, in turn, apoptotic cell death of insulin-secreting cells.

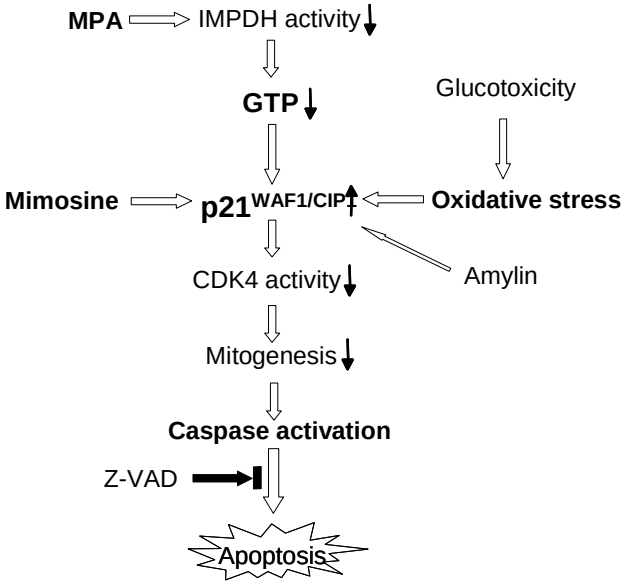

Fig. 31. Schematic diagram for a role of $p21^{WAF1/CIP1}$ in β-cell growth and death. Inhibition of IMPDH by MPA causes GTP depletion resulting in p53-independent induction of $p21^{WAF1/CIP1}$. Oxidative stress due to glucose toxicity and other hostile challenges may also induce $p21^{WAF1/CIP1}$. Elevation of $p21^{WAF1/CIP1}$ may inhibit CDKs such as CDK4 which is essential for b-cell survival. This will suppress mitogenesis which in turn lead to caspase activation and triggers apoptosis as demonstrated in the present study. Amylin may induce apoptosis of b-cells by enhancing expression of $p21^{WAF1/CIP1}$ Mimosine mimics MPA effects to increase $p21^{WAF1/CIP1}$ and promote apoptosis which is blocked by a pan-caspase inhibitor.

4.4. Future work

The results from this work enriched our understanding of the molecular mechanism whereby GTP depletion induces apoptosis of islet β-cells. We found that activation of caspase-2 subsequent to inhibition of mitogenesis plays a critical role in this scenario. Furthermore, our study on cell cycle regulators suggested that $p21^{WAF1/CIP1}$ accumulation may be an upstream signal which leads to cell cycle arrest, caspase activation and eventually apoptosis in our experimental system. However, several important issues (below) remain to be addressed for the thorough elucidation of how GTP depletion causes apoptotic cell death.

1). We have found that GTP depletion resulted in an up-regulation of $p21^{WAF1/CIP1}$, but not p53 and $p27^{KIP1}$; these cell cycle regulators are critical checkpoint target proteins which may determine either cell growth or cell cycle arrest to apoptosis.

Our notion that an up-regulation of $p21^{WAF1/CIP1}$ is closely associated with caspase activation during GTP depletion induced apoptosis of β-cells, however, is based on the indirect observations, i.e. by comparing the time course of the two events and by using a $p21^{WAF1/CIP1}$ inducer. Therefore, more direct evidence is required for ascertaining the existence of such causal relationship between $p21^{WAF1/CIP1}$ and caspase activation during GTP depletion. One means to achieve this would be to selectively suppress or knock down $p21^{WAF1/CIP1}$ and then to examine MPA-induced caspase activation and apoptosis. Unfortunately, no $p21^{WAF1/CIP1}$ inhibitor is available. However, using molecular biology tools (transfection of cells with either antisense oligonucleotides or siRNA of $p21^{WAF1/CIP1}$) to explore this interesting hypothesis seems to be feasible; such experiments are currently in progress.

2). MPA treatment can lower cellular GTP to levels which would impede the function of GTP-binding proteins (Kowluru et al., 2002). Our earlier study revealed that interference with small G-proteins by blockade of their isoprenylation is able to induce β-cell apoptosis. In addition, inactivation of the Rho subfamily of G-proteins by *Clostridium difficile* toxin B potentiates MPA-induced β-cell apoptosis (Li et al., 1998). Therefore, intervention of a small G-protein may mediate between GTP depletion and the events initiating cell death. However, it appears that Rac1 (a member of Rho subfamily proteins) is not involved in this process, since transfection of either a dominant-negative or -positive Rac1 mutant in β-cells did not alter MPA-induced apoptosis (data not shown). Thus, other G-proteins, e.g. Cdc42 and Ras (Kowluru and Morgan, 2002), should be targeted.

3). At the late stage of apoptosis, activation of executive caspases plays an essential role in the degradation of critical proteins, leading to cell destruction. Although

caspase-3 is a player of such function in many circumstances, a specific inhibitor of caspase-3 could not prevent MPA-induced apoptosis of HIT cells in the current study. Therefore, other executive caspases should be investigated. Moreover, a possibility of the participation of other proteolytic enzymes in the execution of cell death in our experimental system, such as calpains (similar to caspases, also a family of cysteine proteases**)** (Chan and Mattson, 1999), granzymes (a family of serine proteases) (Trapani, 2001), and even the ubiquitin-proteasome (Almond and Cohen, 2002) is also required for investigation in the future study.

4). In the study of the role of tTG in GTP depletion induced apoptosis of β-cells, we observed a close correlation between the increase of tTG activity and the induction of apoptosis. In addition, there is evidence that the former might precede the latter. However, two widely-used tTG inhibitors failed to rescue the cells from death, which might be due to the fact that the inhibitors were only able to suppress part of the elevated tTG activity. Although lowering Ca^{2+} was capable of blocking most of the augmented tTG activity due to GTP depletion, this maneuver might have other non-selective effects (e.g. cell morphology) which could complicate MPA action. Therefore, finding a new way to complete remove tTG activity is required to make a solid conclusion about the role of tTG in apoptosis of β-cells induced by GTP depletion. This goal is probably achievable by knocking down tTG through transfection of cells with its antisense oligonucleotides or siRNA.

REFERENCES

Aeschlimann,D., Koeller,M.K., Allen-Hoffmann,B.L., and Mosher,D.F. (1998). Isolation of a cDNA encoding a novel member of the transglutaminase gene family from human keratinocytes. Detection and identification of transglutaminase gene products based on reverse transcription-polymerase chain reaction with degenerate primers. J. Biol. Chem. *273*, 3452-3460.

Aeschlimann,D. and Paulsson,M. (1994). Transglutaminases: protein cross-linking enzymes in tissues and body fluids. Thromb. Haemost. *71*, 402-415.

Agarwal,M.L., Agarwal,A., Taylor,W.R., and Stark,G.R. (1995). p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proc. Natl. Acad. Sci. U. S. A. *92*, 8493-8497.

Alessandrini,A., Chiaur,D.S., and Pagano,M. (1997). Regulation of the cyclin-dependent kinase inhibitor p27 by degradation and phosphorylation. Leukemia. *11*, 342-345.

Allison,A.C. (2000). Immunosuppressive drugs: the first 50 years and a glance forward. Immunopharmacology. *47*, 63-83.

Allison,A.C. and Eugui,E.M. (2000). Mycophenolate mofetil and its mechanisms of action. Immunopharmacology. *47*, 85-118.

Almond,J.B.; Cohen,G.M. The proteasome: a novel target for cancer chemotherapy. Leukemia. *16*, 433-443

Alnemri,E.S., Livingston,D.J., Nicholson,D.W., Salvesen,G., Thornberry,N.A., Wong,W.W., and Yuan,J. (1996). Human ICE/CED-3 protease nomenclature. Cell. *87*, 171.

Alpan,R.S. and Pardee,A.B. (1996). p21WAF1/CIP1/SDI1 is elevated through a p53-independent pathway by mimosine. Cell Growth Differ. *7*, 893-901.

An,B., Goldfarb,R.H., Siman,R., and Dou,Q.P. (1998). Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. Cell Death. Differ. *5*, 1062-1075.

Asfari,M.; Janjic,D.; Meda,P.; Li,G.; Halban,P.A.and Wollheim,C.B. (1992). Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. Endocrinology. *130*, 167-178.

Autuori,F., Farrace,M.G., Oliverio,S., Piredda,L., and Piacentini,M. (1998). "Tissue" transglutaminase and apoptosis. Adv. Biochem. Eng Biotechnol. *62*, 129-136.

Azim,A.C., Marfatia,S.M., Korsgren,C., Dotimas,E., Cohen,C.M., and Chishti,A.H. (1996). Human erythrocyte dematin and protein 4.2 (pallidin) are ATP binding proteins. Biochemistry. *35*, 3001-3006.

Balklava,Z.; Verderio,E.; Collighan,R.; Gross,S.; Adams,J. and Griffin,M. (2002). Analysis of tissue transglutaminase function in the migration of Swiss 3T3 fibroblasts: the active-state conformation of the enzyme does not affect cell motility but is important for its secretion. J.Biol.Chem. 277, 16567-16575

Banki,K., Hutter,E., Gonchoroff,N.J., and Perl,A. (1999). Elevation of mitochondrial transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspases in Fas signaling. J. Immunol. *162*, 1466-1479.

Benchimol,S. (2001). p53-dependent pathways of apoptosis. Cell Death Differ. *8*, 1049-1051.

Bernassola,F., Rossi,A., and Melino,G. (1999). Regulation of transglutaminases by nitric oxide. Ann. N. Y. Acad. Sci. *887*, 83-91.

Bernassola,F.; Federici,M.; Corazzari,M.; Terrinoni,A.; Hribal,M.L.; De,Laurenzi,V; Ranalli,M.; Massa,O.; Sesti,G.; McLean,W.H.; Citro,G.; Barbetti,F.; Melino,G.(2002). Role of transglutaminase 2 in glucose tolerance: knockout mice studies and a putative mutation in a MODY patient. FASEB J. 16, 11, 1371-1378.

Bertalanffy,P., Dubsky,P., Wolner,E., and Weigel,G. (1999). Alterations of endothelial nucleotide levels by mycophenolic acid result in changes of membrane glycosylation and E-selectin expression. Clin. Chem. Lab Med. *37*, 259-264.

Bissonnette,N. and Hunting,D.J. (1998). p21-induced cycle arrest in G1 protects cells from apoptosis induced by UV-irradiation or RNA polymerase II blockage. Oncogene. *16*, 3461-3469.

Blankenberg,F.G., Tait,J., Ohtsuki,K., and Strauss,H.W. (2000). Apoptosis: the importance of nuclear medicine. Nucl. Med. Commun. *21*, 241-250.

Boehm,J.E., Singh,U., Coombs,C., Antonyak,M.A., and Cerione,R.A. (2002). Tissue transglutaminase protects against apoptosis by modifying the tumor suppressor protein p110 Rb. J. Biol. Chem. *277*, 20127-20130

Borner,C. and Monney,L. (1999). Apoptosis without caspases: an inefficient molecular guillotine? Cell Death Differ. *6*, 497-507.

Boulaire,J., Fotedar,A., and Fotedar,R. (2000). The functions of the cdk-cyclin kinase inhibitor p21WAF1. Pathol. Biol. (Paris) *48*, 190-202.

Bowen, I.D, Bowen, S.M. and Jones, A.H. (1998). Mitosis and apoptosis: matters of life and death. Editted by Bowen, I.D.; Published by Chapman & Hall, London

Bras,A., Ruiz-Vela,A., Gonzalez,d.B., and Martinez,A. (1999). Caspase activation by BCR cross-linking in immature B cells: differential effects on growth arrest and apoptosis. FASEB J. *13*, 931-944.

Breckenridge,D.G., Nguyen,M., Kuppig,S., Reth,M., and Shore,G.C. (2002). The procaspase-8 isoform, procaspase-8L, recruited to the BAP31 complex at the endoplasmic reticulum. Proc. Natl. Acad. Sci. U. S. A. *99*, 4331-4336.

Budihardjo,I., Oliver,H., Lutter,M., Luo,X., and Wang,X. (1999). Biochemical pathways of caspase activation during apoptosis. Annu. Rev. Cell Dev. Biol. *15*, 269-290.

Bungay,P.J., Potter,J.M., and Griffin,M. (1984). The inhibition of glucose-stimulated insulin secretion by primary amines. A role for transglutaminase in the secretory mechanism. Biochem. J. *219*, 819-827.

Bungay,P.J.; Owen,R.A.; Coutts,I.C.; Griffin,M. (1986). A role for transglutaminase in glucose-stimulated insulin release from the pancreatic beta-cell. Biochem.J., 235, 1, 269-278.

Bunz,F., Dutriaux,A., Lengauer,C., Waldman,T., Zhou,S., Brown,J.P., Sedivy,J.M., Kinzler,K.W., and Vogelstein,B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science. *282*, 1497-1501.

Buttke,T.M., McCubrey,J.A., and Owen,T.C. (1993). Use of an aqueous soluble tetrazolium/formazan assay to measure viability and proliferation of lymphokine-dependent cell lines. J. Immunol. Methods. *157*, 233-240.

Carr,S.F., Papp,E., Wu,J.C., and Natsumeda,Y. (1993). Characterization of human type I and type II IMP dehydrogenases. J. Biol. Chem. *268*, 27286-27290.

Carter,S.B., Franklin,T.J., Jones,D.F., Leonard,B.J., Mills,S.D., Turner,R.W., and Turner,W.B. (1969). Mycophenolic acid: an anti-cancer compound with unusual properties. Nature. *223*, 848-850.

Catapano,C.V., Dayton,J.S., Mitchell,B.S., and Fernandes,D.J. (1995). GTP depletion induced by IMP dehydrogenase inhibitors blocks RNA-primed DNA synthesis. Mol. Pharmacol. *47*, 948-955.

Chai,F., Evdokiou,A., Young,G.P., and Zalewski,P.D. (2000). Involvement of p21(Waf1/Cip1) and its cleavage by DEVD-caspase during apoptosis of colorectal cancer cells induced by butyrate. Carcinogenesis. *21*, 7-14.

Chan,S.L.; Mattson,M.P. Caspase and calpain substrates: roles in synaptic plasticity and cell death. J.Neurosci.Res. *58*, 167-194

Chen,W., Wang,H.G., Srinivasula,S.M., Alnemri,E.S., and Cooper,N.R. (1999). B cell apoptosis triggered by antigen receptor ligation proceeds via a novel caspase-dependent pathway. J. Immunol. *163*, 2483-2491.

Chen,X., Ko,L.J., Jayaraman,L., and Prives,C. (1996). p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev. *10*, 2438-2451.

Chiarugi,V., Magnelli,L., Cinelli,M., and Basi,G. (1994). Apoptosis and the cell cycle. Cell Mol. Biol. Res. *40*, 603-612.

Choi,K.S., Lim,I.K., Brady,J.N., and Kim,S.J. (1998). ICE-like protease (caspase) is involved in transforming growth factor β1-mediated apoptosis in FaO rat hepatoma cell line. Hepatology. *27*, 415-421.

Cohen,C.M., Dotimas,E., and Korsgren,C. (1993). Human erythrocyte membrane protein band 4.2 (pallidin). Semin. Hematol. *30*, 119-137.

Cohen,M.B., Maybanum,J., and Sadee,W. (1981). Guanine nucleotide depletion and toxicity in mouse T lymphoma (S-49) cells. J. Biol. Chem. *256*, 8713-8717.

Cohn,R.G., Mirkovich,A., Dunlap,B., Burton,P., Chiu,S.H., Eugui,E., and Caulfield,J.P. (1999). Mycophenolic acid increases apoptosis, lysosomes and lipid droplets in human lymphoid and monocytic cell lines. Transplantation. *68*, 411-418.

Cooper,A.J., Wang,J., Pasternack,R., Fuchsbauer,H.L., Sheu,R.K., and Blass,J.P. (2000). Lysine-rich histone (H1) is a lysyl substrate of tissue transglutaminase: possible involvement of transglutaminase in the formation of nuclear aggregates in (CAG)(n)/Q(n) expansion diseases. Dev. Neurosci. *22*, 404-417.

Cory,A.H., Owen,T.C., Barltrop,J.A., and Cory,J.G. (1991). Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun. *3*, 207-212.

Cox,L.S. and Lane,D.P. (1995). Tumour suppressors, kinases and clamps: how p53 regulates the cell cycle in response to DNA damage. Bioessays. *17*, 501-508.

Cryns,V. and Yuan,J. (1998). Proteases to die for. Genes Dev. *12*, 1551-1570.

De, Laurenzi., V and Melino,G. (2001). Gene disruption of tissue transglutaminase. Mol. Cell Biol. *21*, 148-155.

Delaney,C.A., Pavlovic,D., Hoorens,A., Pipeleers,D.G., and Eizirik,D.L. (1997). Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. Endocrinology. *138*, 2610-2614.

Deng,C., Zhang,P., Harper,J.W., Elledge,S.J., and Leder,P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell. *82*, 675-684.

Detimary,P., Xiao,C., and Henquin,J.C. (1997). Tight links between adenine and guanine nucleotide pools in mouse pancreatic islets: a study with mycophenolic acid. Biochem. J. *324 (Pt 2)*, 467-471.

Digits,J.A. and Hedstrom,L. (2000). Drug selectivity is determined by coupling across the NAD+ site of IMP dehydrogenase. Biochemistry. *39*, 1771-1777.

Dinsdale,D., Zhuang,J., and Cohen,G.M. (1999). Redistribution of cytochrome c precedes the caspase-dependent formation of ultracondensed mitochondria, with a reduced inner membrane potential, in apoptotic monocytes. Am. J. Pathol. *155*, 607-618.

Duan,H. and Dixit,V.M. (1997). RAIDD is a new 'death' adaptor molecule. Nature. *385*, 86-89.

Dvorcakova,M.; Macejova,D.; Pallet,V.; Higueret,P.; Vasson,M.P.; Rock,E. and Brtko,J. Transglutamines and endocrine system (minireview). Endocr.Regul. 36, 1, 31-36.

Earnshaw,W.C., Martins,L.M., and Kaufmann,S.H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. *68*, 383-424.

Ekert,P.G.; Silke,J.; Vaux,D.L. (1999). Caspase inhibitors. Cell Death Differ. *6*, 1081-1086

El Deiry,W.S., Harper,J.W., O'Connor,P.M., Velculescu,V.E., Canman,C.E., Jackman,J., Pietenpol,J.A., Burrell,M., Hill,D.E., Wang,Y., and . (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. *54*, 1169-1174.

El Deiry,W.S., Tokino,T., Velculescu,V.E., Levy,D.B., Parsons,R., Trent,J.M., Lin,D., Mercer,W.E., Kinzler,K.W., and Vogelstein,B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell. *75*, 817-825.

Escobar-Henriques,M. and Daignan-Fornier,B. (2001). Transcriptional regulation of the yeast gmp synthesis pathway by its end products. J. Biol. Chem. *276*, 1523-1530.

Fabbi,M., Marimpietri,D., Martini,S., Brancolini,C., Amoresano,A., Scaloni,A., Bargellesi,A., and Cosulich,E. (1999). Tissue transglutaminase is a caspase substrate during apoptosis. Cleavage causes loss of transamidating function and is a biochemical marker of caspase 3 activation. Cell Death. Differ. *6*, 992-1001.

Fau,D., Lekehal,M., Farrell,G., Moreau,A., Moulis,C., Feldmann,G., Haouzi,D., and Pessayre,D. (1997). Diterpenoids from germander, an herbal medicine, induce apoptosis in isolated rat hepatocytes. Gastroenterology. *113*, 1334-1346.

Feng,J.F., Readon,M., Yadav,S.P., and Im,M.J. (1999). Calreticulin down-regulates both GTP binding and transglutaminase activities of transglutaminase II. Biochemistry.*38*, 10743-10749.

Feng,J.F., Rhee,S.G., and Im,M.J. (1996). Evidence that phospholipase delta1 is the effector in the Gh (transglutaminase II)-mediated signaling. J. Biol. Chem. *271*, 16451-16454.

Fero,M.L., Rivkin,M., Tasch,M., Porter,P., Carow,C.E., Firpo,E., Polyak,K., Tsai,L.H., Broudy,V., Perlmutter,R.M., Kaushansky,K., and Roberts,J.M. (1996). A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. Cell. *85*, 733-744.

Ferri,K.F. and Kroemer,G. (2001). Organelle-specific initiation of cell death pathways. Nat. Cell Biol. *3*, E255-E263.

Fesus,L. (1998). Transglutaminase-catalyzed protein cross-linking in the molecular program of apoptosis and its relationship to neuronal processes. Cell Mol. Neurobiol. *18*, 683-694.

Fesus,L., Szucs,E.F., Barrett,K.E., Metcalfe,D.D., and Folk,J.E. (1985). Activation of transglutaminase and production of protein-bound gamma- glutamylhistamine in stimulated mouse mast cells. J. Biol. Chem. *260*, 13771-13778.

Fesus,L. and Thomazy,V. (1988). Searching for the function of tissue transglutaminase: its possible involvement in the biochemical pathway of programmed cell death. Adv. Exp. Med. Biol. *231*, 119-134.

Fesus,L., Thomazy,V., Autuori,F., Ceru,M.P., Tarcsa,E., and Piacentini,M. (1989). Apoptotic hepatocytes become insoluble in detergents and chaotropic agents as a result of transglutaminase action. FEBS Lett. *245*, 150-154.

Flores-Rozas,H., Kelman,Z., Dean,F.B., Pan,Z.Q., Harper,J.W., Elledge,S.J., O'Donnell,M., and Hurwitz,J. (1994). Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. Proc. Natl. Acad. Sci. U. S. A. *91*, 8655-8659.

Folk,J.E. (1980). Transglutaminases. Annu. Rev. Biochem. *49*, 517-531.

Folk,J.E., Park,M.H., Chung,S.I., Schrode,J., Lester,E.P., and Cooper,H.L. (1980). Polyamines as physiological substrates for transglutaminases. J. Biol. Chem. *255*, 3695-3700.

Franchetti,P. and Grifantini,M. (1999). Nucleoside and non-nucleoside IMP dehydrogenase inhibitors as antitumor and antiviral agents. Curr. Med. Chem. *6*, 599-614.

Franklin,T.J. and Cook,J.M. (1969). The inhibition of nucleic acid synthesis by mycophenolic acid. Biochem. J. *113*, 515-524.

Fulton,B. and Markham,A. (1996). Mycophenolate mofetil. A review of its pharmacodynamic and pharmacokinetic properties and clinical efficacy in renal transplantation. Drugs. *51*, 278-298.

Furuya,Y., Lundmo,P., Short,A.D., Gill,D.L., and Isaacs,J.T. (1994). The role of calcium, pH, and cell proliferation in the programmed (apoptotic) death of androgen-independent prostatic cancer cells induced by thapsigargin. Cancer Res. *54*, 6167-6175.

Gamen,S., Anel,A., Lasierra,P., Alava,M.A., Martinez-Lorenzo,M.J., Pineiro,A., and Naval,J. (1997). Doxorubicin-induced apoptosis in human T-cell leukemia is mediated by caspase-3 activation in a Fas-independent way. FEBS Lett. *417*, 360-364.

Gartel,A.L., Serfas,M.S., and Tyner,A.L. (1996). p21--negative regulator of the cell cycle. Proc. Soc. Exp. Biol. Med. *213*, 138-149.

Gentile,V., Thomazy,V., Piacentini,M., Fesus,L., and Davies,P.J. (1992). Expression of tissue transglutaminase in Balb-C 3T3 fibroblasts: effects on cellular morphology and adhesion. J. Cell Biol. *119*, 463-474.

Gervais,J.L., Seth,P., and Zhang,H. (1998). Cleavage of CDK inhibitor p21(Cip1/Waf1) by caspases is an early event during DNA damage-induced apoptosis. J. Biol. Chem. *273*, 19207-19212.

Gilbert,D.M., Neilson,A., Miyazawa,H., DePamphilis,M.L., and Burhans,W.C. (1995). Mimosine arrests DNA synthesis at replication forks by inhibiting deoxyribonucleotide metabolism. J. Biol. Chem. *270*, 9597-9606.

Glesne,D.A., Collart,F.R., and Huberman,E. (1991). Regulation of IMP dehydrogenase gene expression by its end products, guanine nucleotides. Mol. Cell Biol. *11*, 5417-5425.

Gomis,R.; Sener,A.; Malaisse-Lagae,F. and Malaisse,W.J. (1983). Transglutaminase activity in pancreatic islets. Biochim.Biophys.Acta. 760 (3), 384-388

Gomis,R., Arbos,M.A., Sener,A., and Malaisse,W.J. (1986). Glucose-induced activation of transglutaminase in pancreatic islets. Diabetes Res. *3*, 115-117.

Green,D.R. and Reed,J.C. (1998). Mitochondria and apoptosis. Science. *281*, 1309-1312.

Greenberg,C.S., Birckbichler,P.J., and Rice,R.H. (1991). Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. FASEB J. *5*, 3071-3077.

Gross,S.R.; Balklava,Z.; Griffin,M. (2003). Importance of tissue transglutaminase in repair of extracellular matrices and cell death of dermal fibroblasts after exposure to a solarium ultraviolet A source. J.Invest Dermatol. 121, 412-423.

Grusch,M., Rosenberger,G., Fuhrmann,G., Braun,K., Titscher,B., Szekeres,T., Fritzer-Skekeres,M., Oberhuber,G., Krohn,K., Hengstschlaeger,M., Krupitza,G., and Jayaram,H.N. (1999). Benzamide riboside induces apoptosis independent of Cdc25A expression in human ovarian carcinoma N.1 cells. Cell Death. Differ. *6*, 736-744.

Gaudry,C.A.; Verderio,E.; Jones,R.A.; Smith,C. and Griffin,M. (1999). Tissue transglutaminase is an important player at the surface of human endothelial cells: evidence for its externalization and its colocalization with the beta (1) integrin. Exp.Cell Res. 252, 104-113.

Gaudry,C.A.; Verderio,E.; Aeschlimann,D.; Cox,A.; Smith,C. and Griffin,M. (1999). Cell surface localization of tissue transglutaminase is dependent on a fibronectin-binding site in its N-terminal beta-sandwich domain. J.Biol.Chem. 274, 30707-30714.

Gu,J.J., Stegmann,S., Gathy,K., Murray,R., Laliberte,J., Ayscue,L. and Mitchell,B.S. Inhibition of T lymphocyte activation in mice heterozygous for loss of the *IMPDH II* gene. J.Clin.Invest. *106*, 599-606.

Guan,K.L., Jenkins,C.W., Li,Y., Nichols,M.A., Wu,X., O'Keefe,C.L., Matera,A.G., and Xiong,Y. (1994). Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. Genes Dev. *8*, 2939-2952.

Guan,K.L., Jenkins,C.W., Li,Y., O'Keefe,C.L., Noh,S., Wu,X., Zariwala,M., Matera,A.G., and Xiong,Y. (1996). Isolation and characterization of p19INK4d, a p16-related inhibitor specific to CDK6 and CDK4. Mol. Biol. Cell. *7*, 57-70.

Halloran,P.F. (1996). Molecular mechanisms of new immunosuppressants. Clin. Transplant. *10*, 118-123.

Hand,D., Campoy,F.J., Clark,S., Fisher,A., and Haynes,L.W. (1993). Activity and distribution of tissue transglutaminase in association with nerve-muscle synapses. J. Neurochem. *61*, 1064-1072.

Hannon,G.J. and Beach,D. (1994). p15INK4B is a potential effector of TGF-β-induced cell cycle arrest. Nature. *371*, 257-261.

Harper,J.W., Elledge,S.J., Keyomarsi,K., Dynlacht,B., Tsai,L.H., Zhang,P., Dobrowolski,S., Bai,C., Connell-Crowley,L., Swindell,E., and . (1995). Inhibition of cyclin-dependent kinases by p21. Mol. Biol. Cell. *6*, 387-400.

Hauser,I.A., Renders,L., Radeke,H.H., Sterzel,R.B., and Goppelt-Struebe,M. (1999). Mycophenolate mofetil inhibits rat and human mesangial cell proliferation by guanosine depletion. Nephrol. Dial. Transplant. *14*, 58-63.

Hengartner,M.O. (2000). The biochemistry of apoptosis. Nature. *407*, 770-776.

Hirai,H., Roussel,M.F., Kato,J.Y., Ashmun,R.A., and Sherr,C.J. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol. Cell Biol. *15*, 2672-2681.

Hofmann,K., Bucher,P., and Tschopp,J. (1997). The CARD domain: a new apoptotic signalling motif. Trends Biochem. Sci. *22*, 155-156.

Holmes,F.E. and Haynes,L.W. (1996). Superactivation of transglutaminase type 2 without change in enzyme level occurs during progressive neurodegeneration in the mnd mouse mutant. Neurosci. Lett. *213*, 185-188.

Huo,J., Luo,R.H., Metz,S.A., and Li,G. (2002). Activation of caspase-2 mediates the apoptosis induced by GTP-depletion in insulin-secreting (HIT-T15) cells. Endocrinology. *143*, 1695-1704.

Igarashi,S., Koide,R., Shimohata,T., Yamada,M., Hayashi,Y., Takano,H., Date,H., Oyake,M., Sato,T., Sato,A., Egawa,S., Ikeuchi,T., Tanaka,H., Nakano,R., Tanaka,K., Hozumi,I., Inuzuka,T., Takahashi,H., and Tsuji,S. (1998). Suppression of aggregate formation and apoptosis by transglutaminase inhibitors in cells expressing truncated DRPLA protein with an expanded polyglutamine stretch. Nat. Genet. *18*, 111-117.

Im,M.J., Russell,M.A., and Feng,J.F. (1997). Transglutaminase II: a new class of GTP-binding protein with new biological functions. Cell Signal. *9*, 477-482.

Ito,A., Uehara,T., Tokumitsu,A., Okuma,Y., and Nomura,Y. (1999). Possible involvement of cytochrome c release and sequential activation of caspases in ceramide-induced apoptosis in SK-N-MC cells. Biochim. Biophys. Acta. *1452*, 263-274.

Iwahashi,H., Hanafusa,T., Eguchi,Y., Nakajima,H., Miyagawa,J., Itoh,N., Tomita,K., Namba,M., Kuwajima,M., Noguchi,T., Tsujimoto,Y., and Matsuzawa,Y. (1996). Cytokine-induced apoptotic cell death in a mouse pancreatic β-cell line: inhibition by Bcl-2. Diabetologia. *39*, 530-536.

Jackson RC, weber G. IMP dehydrogenase, an enzyme linked with proliferation and maliganancy. Nature. 256-331. 1975

Jayaram,H.N., Cooney,D.A., and Grusch,M. (1999). Consequences of IMP dehydrogenase inhibition, and its relationship to cancer and apoptosis. Curr. Med. Chem. *6*, 561-574.

Ji,C., Marnett,L.J., and Pietenpol,J.A. (1997). Cell cycle re-entry following chemically-induced cell cycle synchronization leads to elevated p53 and p21 protein levels. Oncogene. *15*, 2749-2753.

Johnson,D.E. (2000). Noncaspase proteases in apoptosis. Leukemia. *14*, 1695-1703.

Johnson,T.S., Scholfield,C.I., Parry,J., and Griffin,M. (1998). Induction of tissue transglutaminase by dexamethasone: its correlation to receptor number and transglutaminase-mediated cell death in a series of malignant hamster fibrosarcomas. Biochem. J. *331 (Pt 1)*, 105-112.

Kaneto,H., Kajimoto,Y., Fujitani,Y., Matsuoka,T., Sakamoto,K., Matsuhisa,M., Yamasaki,Y., and Hori,M. (1999a). Oxidative stress induces p21 expression in pancreatic islet cells: possible implication in β-cell dysfunction. Diabetologia. *42*, 1093-1097.

Kaneto,H., Kajimoto,Y., Miyagawa,J., Matsuoka,T., Fujitani,Y., Umayahara,Y., Hanafusa,T., Matsuzawa,Y., Yamasaki,Y., and Hori,M. (1999b). Beneficial effects of antioxidants in diabetes: possible protection of pancreatic β-cells against glucose toxicity. Diabetes. *48*, 2398-2406.

Karlsen,A.E., Pavlovic,D., Nielsen,K., Jensen,J., Andersen,H.U., Pociot,F., Mandrup-Poulsen,T., Eizirik,D.L., and Nerup,J. (2000). Interferon-gamma induces interleukin-1 converting enzyme expression in pancreatic islets by an interferon regulatory factor-1-dependent mechanism. J. Clin. Endocrinol. Metab. *85*, 830-836.

Kastan,M.B., Zhan,Q., El Deiry,W.S., Carrier,F., Jacks,T., Walsh,W.V., Plunkett,B.S., Vogelstein,B., and Fornace,A.J., Jr. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell. *71*, 587-597.

Kay,T.W., Thomas,H.E., Harrison,L.C., and Allison,I. (2000). The B Cell in Autoimmune Diabetes: Many Mechanisms and Pathways of Loss. Trends Endocrinol. Metab. *11*, 11-15.

Kelly,K.J.; Plotkin,Z.; Vulgamott,S.L. and Dagher,P.C. (2003). P53 Mediates the Apoptotic Response to GTP Depletion after Renal Ischemia-Reperfusion: Protective Role of a p53 Inhibitor. J.Am.Soc.Nephrol. *14*, 128-138

Kerr,J.F., Wyllie,A.H., and Currie,A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer. *26*, 239-257.

Kim,I.G., Gorman,J.J., Park,S.C., Chung,S.I., and Steinert,P.M. (1993). The deduced sequence of the novel protransglutaminase E (TGase3) of human and mouse. J. Biol. Chem. *268*, 12682-12690.

Kim,S.Y., Chung,S.I., and Steinert,P.M. (1995). Highly active soluble processed forms of the transglutaminase 1 enzyme in epidermal keratinocytes. J. Biol. Chem. *270*, 18026-18035.

Kiyokawa,H., Kineman,R.D., Manova-Todorova,K.O., Soares,V.C., Hoffman,E.S., Ono,M., Khanam,D., Hayday,A.C., Frohman,L.A., and Koff,A. (1996). Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). Cell. *85*, 721-732.

Kluck,R.M., Bossy-Wetzel,E., Green,D.R., and Newmeyer,D.D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science *275*, 1132-1136.

Knight,C.R., Rees,R.C., Platts,A., Johnson,T., and Griffin,M. (1993). Interleukin-2-activated human effector lymphocytes mediate cytotoxicity by inducing apoptosis in human leukaemia and solid tumour target cells. Immunology. *79*, 535-541.

Koff,A. and Polyak,K. (1995). p27KIP1, an inhibitor of cyclin-dependent kinases. Prog. Cell Cycle Res. *1*, 141-147.

Komatsu,M., Noda,M., and Sharp,G.W. (1998). Nutrient augmentation of Ca^{2+}-dependent and Ca^{2+}-independent pathways in stimulus-coupling to insulin secretion can be distinguished by their guanosine triphosphate requirements: studies on rat pancreatic islets. Endocrinology. *139*, 1172-1183.

Komoriya,A., Packard,B.Z., Brown,M.J., Wu,M.L., and Henkart,P.A. (2000). Assessment of caspase activities in intact apoptotic thymocytes using cell-permeable fluorogenic caspase substrates. J. Exp. Med. *191*, 1819-1828.

Korner,G., Deutsch,V.R., Vlodavsky,I., and Eldor,A. (1993). Effects of ionizing irradiation on endothelial cell transglutaminase. FEBS Lett. *330*, 41-45.

Kowluru,A., Seavey,S.E., Li,G.D., Sorenson,R.L., Weinhaus,A.J., Nesher,R., Rabaglia,M., Vadakekalam,J., and Metz,S.A. (1996). Glucose- and GTP-dependent stimulation of the carboxyl methylation of CDC42 in rodent and human pancreatic islets and pure β cells: evidence for an essential role of GTP-binding proteins in nutrient-induced insulin secretion. J. Clin. Invest. *98*, 540-555.

Kowluru,A.; Morgan,N.G. (2002). GTP-binding proteins in cell survival and demise: the emerging picture in the pancreatic beta-cell. Biochem.Pharmacol. *63*, 1027-1035.

Krude,T. (1999). Mimosine arrests proliferating human cells before onset of DNA replication in a dose-dependent manner. Exp. Cell Res. *247*, 148-159.

Kumar,S. (1999). Mechanisms mediating caspase activation in cell death. Cell Death. Differ. *6*, 1060-1066.

Kwon,S.H., Ahn,S.H., Kim,Y.K., Bae,G.U., Yoon,J.W., Hong,S., Lee,H.Y., Lee,Y.W., Lee,H.W., and Han,J.W. (2002). Apicidin, a histone deacetylase inhibitor, induces apoptosis and Fas/Fas ligand expression in human acute promyelocytic leukemia cells. J. Biol. Chem. *277*, 2073-2080.

Laliberte,J., Yee,A., Xiong,Y., and Mitchell,B.S. (1998). Effects of guanine nucleotide depletion on cell cycle progression in human T lymphocytes. Blood. *91*, 2896-2904.

Lane,J.D., Lucocq,J., Pryde,J., Barr,F.A., Woodman,P.G., Allan,V.J., and Lowe,M. (2002). Caspase-mediated cleavage of the stacking protein GRASP65 is required for Golgi fragmentation during apoptosis. J. Cell Biol. *156*, 495-509.

Lanford,R.E., Chavez,D., Guerra,B., Lau,J.Y., Hong,Z., Brasky,K.M., and Beames,B. (2001). Ribavirin induces error-prone replication of GB virus B in primary tamarin hepatocytes. J. Virol. *75*, 8074-8081.

Li,G.D, Segu,V.B., Rabaglia,M.E., Luo,R.H., Kowluru,A., and Metz,S.A. (1998). Prolonged depletion of guanosine triphosphate induces death of insulin-secreting cells by apoptosis. Endocrinology. *139*, 3752-3762.

Li,G.D., Kowluru,A., and Metz,S.A. (1996). Characterization of prenylcysteine methyltransferase in insulin- secreting cells. Biochem. J. *316*, 345-351.

Li,G.D., Luo,R.H., and Metz,S.A. (2000). Effects of inhibitors of guanine nucleotide synthesis on membrane potential and cytosolic free Ca2+ levels in insulin-secreting cells. Biochem. Pharmacol. *59*, 545-556.

Li,H., Bergeron,L., Cryns,V., Pasternack,M.S., Zhu,H., Shi,L., Greenberg,A., and Yuan,J. (1997a). Activation of caspase-2 in apoptosis. J. Biol. Chem. *272*, 21010-21017.

Li,P., Nijhawan,D., Budihardjo,I., Srinivasula,S.M., Ahmad,M., Alnemri,E.S., and Wang,X. (1997b). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. *91*, 479-489.

Liu,Y., Bohn,S.A., and Sherley,J.L. (1998a). Inosine-5'-monophosphate dehydrogenase is a rate-determining factor for p53-dependent growth regulation. Mol. Biol. Cell. *9*, 15-28.

Liu,Y., Riley,L.B., Bohn,S.A., Boice,J.A., Stadler,P.B., and Sherley,J.L. (1998b). Comparison of bax, waf1, and IMP dehydrogenase regulation in response to wild-type p53 expression under normal growth conditions. J. Cell Physiol. *177*, 364-376.

Lorand,L. and Conrad,S.M. (1984). Transglutaminases. Mol. Cell Biochem. *58*, 9-35.

Lorenzo,H.K., Susin,S.A., Penninger,J., and Kroemer,G. (1999). Apoptosis inducing factor (AIF): a phylogenetically old, caspase- independent effector of cell death. Cell Death. Differ. *6*, 516-524.

Lowe,J.K., Brox,L., and Henderson,J.F. (1977). Consequences of inhibition of guanine nucleotide synthesis by mycophenolic acid and virazole. Cancer Res. *37*, 736-743.

Luchetti,F., Mariani,A.R., Columbaro,M., Di Baldassarre,A., Cinti,C., and Falcieri,E. (1999). Apoptotic pathways depend on the target enzymatic activity and not on the triggering agent. Scanning. *21*, 29-35.

MacFarlane,M., Cain,K., Sun,X.M., Alnemri,E.S., and Cohen,G.M. (1997). Processing/activation of at least four interleukin-1β converting enzyme-like proteases occurs during the execution phase of apoptosis in human monocytic tumor cells. J. Cell Biol. *137*, 469-479.

Mancini,M., Machamer,C.E., Roy,S., Nicholson,D.W., Thornberry,N.A., Casciola-Rosen,L.A., and Rosen,A. (2000). Caspase-2 is localized at the Golgi complex and cleaves golgin-160 during apoptosis. J. Cell Biol. *149*, 603-612.

Martelli,A.M., Zweyer,M., Ochs,R.L., Tazzari,P.L., Tabellini,G., Narducci,P., and Bortul,R. (2001). Nuclear apoptotic changes: an overview. J. Cell Biochem. *82*, 634-646.

Matsumoto,Y., Hayashi,K., and Nishida,E. (1999). Cyclin-dependent kinase 2 (Cdk2) is required for centrosome duplication in mammalian cells. Curr. Biol. *9*, 429-432.

Matsuyama,S. and Reed,J.C. (2000). Mitochondria-dependent apoptosis and cellular pH regulation. Cell Death. Differ. *7*, 1155-1165.

Medema,J.P., Scaffidi,C., Kischkel,F.C., Shevchenko,A., Mann,M., Krammer,P.H., and Peter,M.E. (1997). FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J. *16*, 2794-2804.

Meier,P., Finch,A., and Evan,G. (2000). Apoptosis in development. Nature. *407*, 796-801.

Meikrantz,W. and Schlegel,R. (1995). Apoptosis and the cell cycle. J. Cell Biochem. *58*, 160-174.

Melino,G., Annicchiarico-Petruzzelli,M., Piredda,L., Candi,E., Gentile,V., Davies,P.J., and Piacentini,M. (1994). Tissue transglutaminase and apoptosis: sense and antisense transfection studies with human neuroblastoma cells. Mol. Cell Biol. *14*, 6584-6596.

Melino,G. and Piacentini,M. (1998). 'Tissue' transglutaminase in cell death: a downstream or a multifunctional upstream effector? FEBS Lett. *430*, 59-63.

Melino,G., Thiele,C.J., Knight,R.A., and Piacentini,M. (1997). Retinoids and the control of growth/death decisions in human neuroblastoma cell lines. J. Neurooncol. *31*, 65-83.

Mercer,W.E. (1998). Checking on the cell cycle. J. Cell Biochem. Suppl *30-31*, 50-54.

Meredith,M., Li,G., and Metz,S.A. (1997). Inhibition of calcium-induced insulin secretion from intact HIT-T15 or INS-1 β cells by GTP depletion. Biochem. Pharmacol. *53*, 1873-1882.

Meredith,M., Rabaglia,M., Corbett,J.A., and Metz,S.A. (1996). Dual functional effects of interleukin-1β on purine nucleotides and insulin secretion in rat islets and INS-1 cells. Diabetes. *45*, 1783-1791.

Metz,S., Holland,S., Johnson,L., Espling,E., Rabaglia,M., Segu,V., Brockenbrough,J.S., and Tran,P.O. (2001). Inosine-5'-monophosphate dehydrogenase is required for mitogenic competence of transformed pancreatic β cells. Endocrinology. *142*, 193-204.

Metz,S.A. and Kowluru,A. (1999). Inosine monophosphate dehydrogenase: A molecular switch integrating pleiotropic GTP-dependent β-cell functions. Proc. Assoc. Am. Physicians *111*, 335-346.

Metz,S.A., Rabaglia,M.E., and Pintar,T.J. (1992). Selective inhibitors of GTP synthesis impede exocytotic insulin release from intact rat islets. J. Biol. Chem. *267*, 12517-12527.

Mitchell,B.S., Dayton,J.S., Turka,L.A., and Thompson,C.B. (1993). IMP dehydrogenase inhibitors as immunomodulators. Ann. N. Y. Acad. Sci. *685*, 217-224.

Moller,M.B. (2000). P27 in cell cycle control and cancer. Leuk. Lymphoma. *39*, 19-27.

Nagai,M., Natsumeda,Y., Konno,Y., Hoffman,R., Irino,S., and Weber,G. (1991). Selective up-regulation of type II inosine 5'-monophosphate dehydrogenase messenger RNA expression in human leukemias. Cancer Res. *51*, 3886-3890.

Nagai, M. Natsumeda Y. and Weber G. (2002). Proliferation-linked regulation of type II IMP dehydrogenase gene in human normal lymphocytes and HL-60 leukemic cells. Cancer Research. *52*, 258-261.

Nagy,L., Saydak,M., Shipley,N., Lu,S., Basilion,J.P., Yan,Z.H., Syka,P., Chandraratna,R.A., Stein,J.P., Heyman,R.A., and Davies,P.J. (1996). Identification and characterization of a versatile retinoid response element (retinoic acid receptor response element-retinoid X receptor response element) in the mouse tissue transglutaminase gene promoter. J. Biol. Chem. *271*, 4355-4365.

Nakagawa,T., Zhu,H., Morishima,N., Li,E., Xu,J., Yankner,B.A., and Yuan,J. (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature. *403*, 98-103.

Nakaoka,H., Perez,D.M., Baek,K.J., Das,T., Husain,A., Misono,K., Im,M.J., and Graham,R.M. (1994). Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function. Science. *264*, 1593-1596.

Nakayama,K., Ishida,N., Shirane,M., Inomata,A., Inoue,T., Shishido,N., Horii,I., Loh,D.Y., and Nakayama,K. (1996). Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell. *85*, 707-720.

Nanda,N., Iismaa,S.E., Owens,W.A., Husain,A., Mackay,F., and Graham,R.M. (2001). Targeted inactivation of Gh/tissue transglutaminase II. J. Biol. Chem. *276*, 20673-20678.

Natsumeda,Y. and Carr,S.F. (1993). Human type I and II IMP dehydrogenases as drug targets. Ann. N. Y. Acad. Sci. *696*, 88-93.

Natsumeda, Y., Ikegami, T., Murayama K, and Weber, G. De novo guanylate synthesis in the commitment to replication in hepatoma 3924A cells. Cancer Research. *48*, 507. 1988.

Nguyen,B.T., El Sayed,Y.M., and Sadee,W. (1984). Interaction among the distinct effects of adenine and guanine depletion in mouse lymphoma cells. Cancer Res. *44*, 2272-2277.

Nguyen,B.T. and Sadee,W. (1986). Compartmentation of guanine nucleotide precursors for DNA synthesis. Biochem. J. *234*, 263-269.

Nicoletti,I., Migliorati,G., Pagliacci,M.C., Grignani,F., and Riccardi,C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods. *139*, 271-279.

Noda,A., Ning,Y., Venable,S.F., Pereira-Smith,O.M., and Smith,J.R. (1994). Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp. Cell Res. *211*, 90-98.

O'Connor,P.M. (1997). Mammalian G1 and G2 phase checkpoints. Cancer Surv. *29*, 151-182.

Oliverio,S., Amendola,A., Rodolfo,C., Spinedi,A., and Piacentini,M. (1999). Inhibition of "tissue" transglutaminase increases cell survival by preventing apoptosis. J. Biol. Chem. *274*, 34123-34128.

Ou,H., Haendeler,J., Aebly,M.R., Kelly,L.A., Cholewa,B.C., Koike,G., Kwitek-Black,A., Jacob,H.J., Berk,B.C., and Miano,J.M. (2000). Retinoic acid-induced tissue transglutaminase and apoptosis in vascular smooth muscle cells. Circ. Res. *87*, 881-887.

Ozeki,A. and Tsukamoto,I. (1999). Retinoic acid repressed the expression of c-fos and c-jun and induced apoptosis in regenerating rat liver after partial hepatectomy. Biochim. Biophys. Acta. *1450*, 308-319.

Pall,M.L. (1985). GTP: a central regulator of cellular anabolism. Curr. Top. Cell Regul. *25*, 1-20.

Paraskevas,S., Maysinger,D., Wang,R., Duguid,T.P., and Rosenberg,L. (2000). Cell loss in isolated human islets occurs by apoptosis. Pancreas. *20*, 270-276.

Patel,S.D., Tran,A.C., Ge,Y., Moskalenko,M., Tsui,L., Banik,G., Tom,W., Scott,M., Chen,L., Van Roey,M., Rivkin,M., Mendez,M., Gyuris,J., and McArthur,J.G. (2000). The p53-independent tumoricidal activity of an adenoviral vector encoding a p27-p16 fusion tumor suppressor gene. Mol. Ther. *2*, 161-169.

Pellizzaro,C., Coradini,D., Daniotti,A., Abolafio,G., and Daidone,M.G. (2001). Modulation of cell cycle-related protein expression by sodium butyrate in human non-small cell lung cancer cell lines. Int. J. Cancer. *91*, 654-657.

Piacentini,M., Autuori,F., Dini,L., Farrace,M.G., Ghibelli,L., Piredda,L., and Fesus,L. (1991). "Tissue" transglutaminase is specifically expressed in neonatal rat liver cells undergoing apoptosis upon epidermal growth factor-stimulation. Cell Tissue Res. *263*, 227-235.

Piacentini,M., Farrace,M.G., Piredda,L., Matarrese,P., Ciccosanti,F., Falasca,L., Rodolfo,C., Giammarioli,A.M., Verderio,E., Griffin,M., and Malorni,W. (2002). Transglutaminase

overexpression sensitizes neuronal cell lines to apoptosis by increasing mitochondrial membrane potential and cellular oxidative stress. J. Neurochem. *81*, 1061-1072.

Piacentini,M., Fesus,L., and Melino,G. (1993). Multiple cell cycle access to the apoptotic death programme in human neuroblastoma cells. FEBS Lett. *320*, 150-154.

Piacentini,M., Martinet,N., Beninati,S., and Folk,J.E. (1988). Free and protein-conjugated polyamines in mouse epidermal cells. Effect of high calcium and retinoic acid. J. Biol. Chem. *263*, 3790-3794.

Piacentini,M., Rodolfo,C., Farrace,M.G., and Autuori,F. (2000). "Tissue" transglutaminase in animal development. Int. J. Dev. Biol. *44*, 655-662.

Pick,A., Clark,J., Kubstrup,C., Levisetti,M., Pugh,W., Bonner-Weir,S., and Polonsky,K.S. (1998). Role of apoptosis in failure of β-cell mass compensation for insulin resistance and β-cell defects in the male Zucker diabetic fatty rat. Diabetes. *47*, 358-364.

Piredda,L., Farrace,M.G., Lo,B.M., Malorni,W., Melino,G., Petruzzelli,R., and Piacentini,M. (1999). Identification of 'tissue' transglutaminase binding proteins in neural cells committed to apoptosis. FASEB J. *13*, 355-364.

Polyak,K., Lee,M.H., Erdjument-Bromage,H., Koff,A., Roberts,J.M., Tempst,P., and Massague,J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell. *78*, 59-66.

Rago,R., Mitchen,J., and Wilding,G. (1990). DNA fluorometric assay in 96-well tissue culture plates using Hoechst 33258 after cell lysis by freezing in distilled water. Anal. Biochem. *191*, 31-34.

Rane,S.G., Dubus,P., Mettus,R.V., Galbreath,E.J., Boden,G., Reddy,E.P., and Barbacid,M. (1999). Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in β-islet cell hyperplasia. Nat. Genet. *22*, 44-52.

Rao,R.V., Hermel,E., Castro-Obregon,S., del Rio,G., Ellerby,L.M., Ellerby,H.M., and Bredesen,D.E. (2001). Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. J. Biol. Chem. *276*, 33869-33874.

Rice,R.H. and Green,H. (1977). The cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked protein. Cell. *11*, 417-422.

Ritter,S.J. and Davies,P.J. (1998). Identification of a transforming growth factor-β1/bone morphogenetic protein 4 (TGF-β1/BMP4) response element within the mouse tissue transglutaminase gene promoter. J. Biol. Chem. *273*, 12798-12806.

Roberts,J.M., Koff,A., Polyak,K., Firpo,E., Collins,S., Ohtsubo,M., and Massague,J. (1994). Cyclins, Cdks, and cyclin kinase inhibitors. Cold Spring Harb. Symp. Quant. Biol. *59*, 31-38.

Rosenthal,A.K.; Heinkel,D. and Gohr,C.M. (2003). Thyroxine stimulates transglutaminase activity in articular chondrocytes. Osteoarthritis.Cartilage. 11, 463-470

Schuler,M., Bossy-Wetzel,E., Goldstein,J.C., Fitzgerald,P., and Green,D.R. (2000). p53 induces apoptosis by caspase activation through mitochondrial cytochrome c release. J. Biol. Chem. *275*, 7337-7342.

Segu,V.B., Li,G., and Metz,S.A. (1998). Use of a soluble tetrazolium compound to assay metabolic activation of intact β cells. Metabolism. *47*, 824-830.

Sener,A.; Gomis,R.; Lebrun,P.; Herchuelz,A.; Malaisse-Lagae,F. and Malaisse,W.J. (1984). Methylamine and islet function: possible relationship to Ca2+-sensitive transglutaminase. Mol.Cell Endocrinol. 36 (3), 175-180.

Sener,A.; Dunlop,M.E.; Gomis,R.; Mathias,P.C.; Malaisse-Lagae,F.; Malaisse,W.J.(1985). Role of transglutaminase in insulin release. Study with glycine and sarcosine methylesters. Endocrinology. 117, 1, 237-242

Serrano,M., Hannon,G.J., and Beach,D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature. *366*, 704-707.

Shearwin-Whyatt,L.M., Harvey,N.L., and Kumar,S. (2000). Subcellular localization and CARD-dependent oligomerization of the death adaptor RAIDD. Cell Death. Differ. *7*, 155-165.

Sherley,J.L. (1991). Guanine nucleotide biosynthesis is regulated by the cellular p53 concentration. J. Biol. Chem. *266*, 24815-24828.

Sherley,J.L., Stadler,P.B., and Johnson,D.R. (1995). Expression of the wild-type p53 antioncogene induces guanine nucleotide- dependent stem cell division kinetics. Proc. Natl. Acad. Sci. U. S. A. *92*, 136-140.

Sherr,C.J. (1993). Mammalian G1 cyclins. Cell. *73*, 1059-1065.

Sherr,C.J. and Roberts,J.M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. *13*, 1501-1512.

Shi, Y. A structural view of mitochondria-mediated apoptosis. Nat.Struct.Biol. 8, 394-401.

Shimabukuro,M., Zhou,Y.T., Levi,M., and Unger,R.H. (1998). Fatty acid-induced β cell apoptosis: a link between obesity and diabetes. Proc. Natl. Acad. Sci. U. S. A. *95*, 2498-2502.

Sidi,Y. and Mitchell,B.S. (1984). 2'-deoxyguanosine toxicity for B and mature T lymphoid cell lines is mediated by guanine ribonucleotide accumulation. J. Clin. Invest. *74*, 1640-1648.

Slingerland,J. and Pagano,M. (2000). Regulation of the cdk inhibitor p27 and its deregulation in cancer. J. Cell Physiol. *183*, 10-17.

Smethurst,P.A. and Griffin,M. (1996). Measurement of tissue transglutaminase activity in a permeabilized cell system: its regulation by Ca2+ and nucleotides. Biochem. J. *313 (Pt 3)*, 803-808.

Soane,L., Rus,H., Niculescu,F., and Shin,M.L. (1999). Inhibition of oligodendrocyte apoptosis by sublytic C5b-9 is associated with enhanced synthesis of bcl-2 and mediated by inhibition of caspase- 3 activation. J. Immunol. *163*, 6132-6138.

Soengas,M.S., Alarcon,R.M., Yoshida,H., Giaccia,A.J., Hakem,R., Mak,T.W., and Lowe,S.W. (1999). Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. Science. *284*, 156-159.

Sowa,Y., Orita,T., Hiranabe-Minamikawa,S., Nakano,K., Mizuno,T., Nomura,H., and Sakai,T. (1999). Histone deacetylase inhibitor activates the p21/WAF1/Cip1 gene promoter through the Sp1 sites. Ann. N. Y. Acad. Sci. *886*, 195-199.

Stadler,P.B., Pennacchi,J., and Sherley,J.L. (1994). Inosine-5'-monophosphate dehydrogenase activity is maintained in immortalized murine cells growth-arrested by serum deprivation. Adv. Enzyme Regul. *34*, 91-106.

Stefanis,L., Park,D.S., Friedman,W.J., and Greene,L.A. (1999). Caspase-dependent and -independent death of camptothecin-treated embryonic cortical neurons. J. Neurosci. *19*, 6235-6247.

Steinert,P.M. and Marekov,L.N. (1995). The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross-linked

components of the human epidermal cornified cell envelope. J. Biol. Chem. *270*, 17702-17711.

Stephens,L.A., Thomas,H.E., Ming,L., Grell,M., Darwiche,R., Volodin,L., and Kay,T.W. (1999). Tumor necrosis factor-alpha-activated cell death pathways in NIT-1 insulinoma cells and primary pancreatic β cells. Endocrinology. *140*, 3219-3227.

Strasser,A., O'Connor,L., and Dixit,V.M. (2000). Apoptosis signaling. Annu. Rev. Biochem. *69*, 217-245.

Susin,S.A., Lorenzo,H.K., Zamzami,N., Marzo,I., Brenner,C., Larochette,N., Prevost,M.C., Alzari,P.M., and Kroemer,G. (1999a). Mitochondrial release of caspase-2 and -9 during the apoptotic process. J. Exp. Med. *189*, 381-394.

Susin,S.A., Lorenzo,H.K., Zamzami,N., Marzo,I., Snow,B.E., Brothers,G.M., Mangion,J., Jacotot,E., Costantini,P., Loeffler,M., Larochette,N., Goodlett,D.R., Aebersold,R., Siderovski,D.P., Penninger,J.M., and Kroemer,G. (1999b). Molecular characterization of mitochondrial apoptosis-inducing factor. Nature. *397*, 441-446.

Szondy,Z., Molnar,P., Nemes,Z., Boyiadzis,M., Kedei,N., Toth,R., and Fesus,L. (1997). Differential expression of tissue transglutaminase during in vivo apoptosis of thymocytes induced via distinct signalling pathways. FEBS Lett. *404*, 307-313.

Takizawa,C.G. and Morgan,D.O. (2000). Control of mitosis by changes in the subcellular location of cyclin-B1- Cdk1 and Cdc25C. Curr. Opin. Cell Biol. *12*, 658-665.

Talanian,R.V., Quinlan,C., Trautz,S., Hackett,M.C., Mankovich,J.A., Banach,D., Ghayur,T., Brady,K.D., and Wong,W.W. (1997). Substrate specificities of caspase family proteases. J. Biol. Chem. *272*, 9677-9682.

Taylor,W.R. and Stark,G.R. (2001). Regulation of the G2/M transition by p53. Oncogene. *20*, 1803-1815.

Tepper,A.D., Cock,J.G., de Vries,E., Borst,J., and van Blitterswijk,W.J. (1997). CD95/Fas-induced ceramide formation proceeds with slow kinetics and is not blocked by caspase-3/CPP32 inhibition. J. Biol. Chem. *272*, 24308-24312.

Thome,M., Hofmann,K., Burns,K., Martinon,F., Bodmer,J.L., Mattmann,C., and Tschopp,J. (1998). Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. Curr. Biol. *8*, 885-888.

Thornberry,N.A. and Lazebnik,Y. (1998). Caspases: enemies within. Science. *281*, 1312-1316.

Toyoshima,H. and Hunter,T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell. *78*, 67-74.

Trapani,J.A. (2001). Granzymes: a family of lymphocyte granule serine proteases. Genome Biol. *2,* REVIEWS3014

Trejo-Skalli,A.V., Velasco,P.T., Murthy,S.N., Lorand,L., and Goldman,R.D. (1995). Association of a transglutaminase-related antigen with intermediate filaments. Proc. Natl. Acad. Sci. U. S. A. *92*, 8940-8944.

Tsai,Y.H., Lai,W.F., Wu,Y.W., and Johnson,L.R. (1998). Two distinct classes of rat intestinal mucosal enzymes incorporating putrescine into protein. FEBS Lett. *435*, 251-256.

Tsutsui,T., Hesabi,B., Moons,D.S., Pandolfi,P.P., Hansel,K.S., Koff,A., and Kiyokawa,H. (1999). Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. Mol. Cell Biol. *19*, 7011-7019.

Tucholski,J. and Johnson,G.V. (2002). Tissue transglutaminase differentially modulates apoptosis in a stimuli-dependent manner. J. Neurochem. *81*, 780-791.

Uray,I.P., Davies,P.J., and Fesus,L. (2001). Pharmacological separation of the expression of tissue transglutaminase and apoptosis after chemotherapeutic treatment of HepG2 cells. Mol. Pharmacol. *59*, 1388-1394.

Vallee,S., Fouchier,F., Braguer,D., Marvaldi,J., and Champion,S. (2000). Ribavirin-induced resistance to heat shock, inhibition of the Ras-Raf-1 pathway and arrest in G(1). Eur. J. Pharmacol. *404*, 49-62.

Vander Heiden,M.G., Chandel,N.S., Williamson,E.K., Schumacker,P.T., and Thompson,C.B. (1997). Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell. *91*, 627-637.

Verderio,E.; Gaudry,C.; Gross,S.; Smith,C.; Downes,S. and Griffin,M. (1999). Regulation of cell surface tissue transglutaminase: effects on matrix storage of latent transforming growth factor-beta binding protein-1. J.Histochem.Cytochem. 47, 1417-1432.

Vezza,R., Habib,A., and FitzGerald,G.A. (1999). Differential signaling by the thromboxane receptor isoforms via the novel GTP-binding protein, Gh. J. Biol. Chem. *274*, 12774-12779.

Virag,L. and Szabo,C. (2000). BCL-2 protects peroxynitrite-treated thymocytes from poly(ADP-ribose) synthase (PARS)-independent apoptotic but not from PARS-mediated necrotic cell death. Free Radic. Biol. Med. *29*, 704-713.

Vousden,K.H.and Lu,X. (2002). Live or let die: the cell's response to p53. Nat.Rev.Cancer. *2*, 594-604.

Wakabayashi,T. and Karbowski,M. (2001). Structural changes of mitochondria related to apoptosis. Biol. Signals Recept. *10*, 26-56.

Wang,S., Miura,M., Jung,Y.K., Zhu,H., Li,E., and Yuan,J. (1998). Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. Cell. *92*, 501-509.

Williams,R.H., Lively,D.H., Delong,D.C., Cline,J.C., and Sweeny,M.J. (1968). Mycophenolic acid: antiviral and antitumor properties. J. Antibiot. (Tokyo) *21*, 463-464.

Wong,J.K., Kennedy,P.R., and Belcher,S.M. (2001). Simplified serum- and steroid-free culture conditions for high-throughput viability analysis of primary cultures of cerebellar granule neurons. J. Neurosci. Methods. *110*, 45-55.

Wu,M., Bellas,R.E., Shen,J., and Sonenshein,G.E. (1998). Roles of the tumor suppressor p53 and the cyclin-dependent kinase inhibitor p21WAF1/CIP1 in receptor-mediated apoptosis of WEHI 231 B lymphoma cells. J. Exp. Med. *187*, 1671-1679.

Wyllie,A.H., Kerr,J.F., and Currie,A.R. (1980). Cell death: the significance of apoptosis. Int. Rev. Cytol. *68*, 251-306.

Xiong,Y., Hannon,G.J., Zhang,H., Casso,D., Kobayashi,R., and Beach,D. (1993). p21 is a universal inhibitor of cyclin kinases. Nature. *366*, 701-704.

Xu,Y., Bialik,S., Jones,B.E., Iimuro,Y., Kitsis,R.N., Srinivasan,A., Brenner,D.A., and Czaja,M.J. (1998). NF-kappaB inactivation converts a hepatocyte cell line TNF-alpha response from proliferation to apoptosis. Am. J. Physiol. *275*, C1058-C1066.

Xue,D., Shaham,S., and Horvitz,H.R. (1996). The Caenorhabditis elegans cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. Genes Dev. *10*, 1073-1083.

Yalowitz,J.A. and Jayaram,H.N. (2000). Molecular targets of guanine nucleotides in differentiation, proliferation and apoptosis. Anticancer Res. *20*, 2329-2338.

Yang,J., Liu,X., Bhalla,K., Kim,C.N., Ibrado,A.M., Cai,J., Peng,T.I., Jones,D.P., and Wang,X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science. *275*, 1129-1132.

Yuan,J., Shaham,S., Ledoux,S., Ellis,H.M., and Horvitz,H.R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 β-converting enzyme. Cell. *75*, 641-652.

Zetterberg,A., Larsson,O., and Wiman,K.G. (1995). What is the restriction point? Curr. Opin. Cell Biol. *7*, 835-842.

Zhang,H., Koty,P.P., Mayotte,J., and Levitt,M.L. (1999a). Induction of multiple programmed cell death pathways by IFN-β in human non-small-cell lung cancer cell lines. Exp. Cell Res. *247*, 133-141.

Zhang,J., Lesort,M., Guttmann,R.P., and Johnson,G.V. (1998). Modulation of the in situ activity of tissue transglutaminase by calcium and GTP1. J. Biol. Chem. *273*, 2288-2295.

Zhang,S., Liu,J., Saafi,E.L., and Cooper,G.J. (1999b). Induction of apoptosis by human amylin in RINm5F islet betaβ-cells is associated with enhanced expression of p53 and p21WAF1/CIP1. FEBS Lett. *455*, 315-320.

Zi,X. and Agarwal,R. (1999). Silibinin decreases prostate-specific antigen with cell growth inhibition via G1 arrest, leading to differentiation of prostate carcinoma cells: implications for prostate cancer intervention. Proc. Natl. Acad. Sci. U. S. A. *96*, 7490-7495